Characterization of Multicompartmental Microparticles for Cochlear Drug Delivery

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biomedical Engineering)
in The University of Michigan
2014

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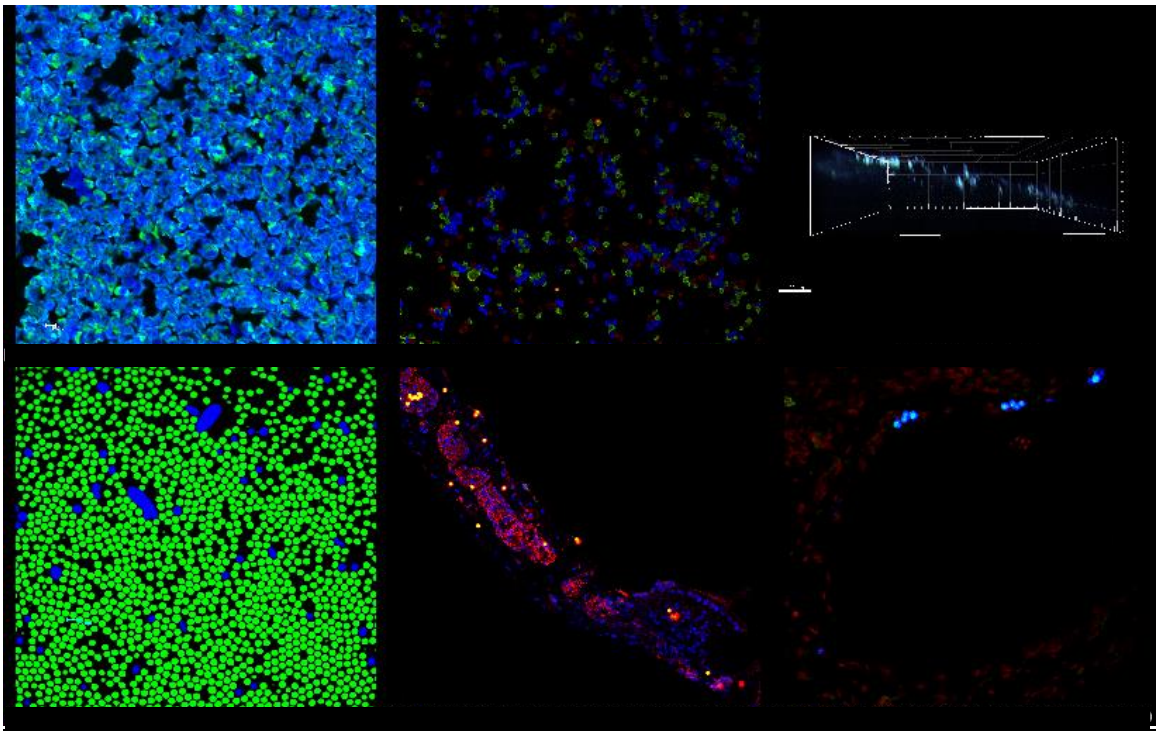

Dedication

This dissertation is dedicated with love to the memories of

Ms. Ruby Grace Jones

Mrs. Ida Dale Ross

Mr. LD Ross Sr.

Mr. LaMont Toliver

Ms. Imade Asemota

Acknowledgements

I am eternally indebted and grateful to many persons who have played integral roles in my success to this point. I would like to convey my sincere thanks to each person including those who may not be singled out in the paper. I would first like to thank all mighty God for giving me the spiritual steadfastness that was needed as I've undertaken this incredible academic journey. Whenever I would encounter turbulence in the process I always found comfort in prayer.

Many persons had significant roles in my scientific development in graduate school. I have the highest regards for Professor Altschuler my mentor and dissertation committee chair. He gave me a chance and I am honored to have been a part of his lab. His patient demeanor and scholarly aptitude were critical to my success in the PhD process. I had the opportunity to work with other outstanding professionals in the lab. Diane Prieskorn was incredible in providing assistance and sharing her expertise with animal surgeries and ABRs. Noel Wys and Ariane Kanicki provided immeasurable help with the surface preparations and perfusions for this project. I am indebted to Cathy Martin for her wonderful work with the cytocochleograms and Susan Deremer for the outstanding job she did with the ABRs. I want to thank Jong Kim for his support in ordering the supplies and providing company for me on many weekends in the lab. I would like to recognize and express my heartfelt thanks to my dissertation committee, Professor Lahann, Professor Miller, Professor Sept, and Professor Grosh. Your feedback and collaboration

were critical in guiding me through the dissertation process. Sahar Rahmani from the Lahann Lab is lauded for her invaluable contributions in providing the microparticles used for this work. I would be remiss if I didn't thank the administrative staff at Kresge Hearing Research Institute for their tremendous work in ordering supplies and assisting with funding applications.

It is very important for me to acknowledge the importance of my family, community, and friends in the PhD journey. My parents LD Ross Jr. and Annetta M. Ross have been guiding lights and pillars of strength for me throughout the process. Aftin, my identical twin sister whom I began this journey with, has been an amazing influence and has helped me each step of the way with her positive encouragement and advice. I would also like to thank my siblings, grandparents, aunts, uncles, and cousins for all of their prayers and encouragement. My family has been instrumental in my success and have provided "beacons" of hope throughout my graduate years. The Michigan community was a very important component of the graduate school experience because it allowed me to be ingratiated into a larger community of support. I am grateful to have been a member and an officer in The Society of Minority Engineers and Scientists Graduate Component (SMES-G), Movement for Underrepresented Sisters in Science and Engineering (MUSES), and Alliance for Graduate Education and the Professoriate (AGEP). During my time in Ann Arbor, I joined the Bethel AME Church. I want to thank my church family, pastor Dr. Joseph Cousin and Reverend Yolanda Whiten and the rest of the congregants for their unwavering support. In addition to these wonderful organizations, I have a group of friends that have helped me to enjoy the experience and keep things in perspective. Thanks Kyla, Angelique, Taeyjuana, Gamola, Jamie, and Afi for your

friendship, presentation assistance, and writing review. Joy and Mia, you were a great set of “ears” allowing me to vent and then sharing your words of encouragement with me. Thanks to Mustapha, Michael L., Gbenga, Memie, Korey, and Brian B. for being there for me throughout the years. A special thanks to Dean Gallimore for your advice and support throughout this process.

I would also like to thank the professors and staff at the University of Maryland Baltimore County (UMBC) for preparing me for the rigors of graduate school. I am particularly grateful to the administrators and staff of the Meyerhoff Scholars Program of which I was a member during my undergraduate years. Your insistence on hard work and giving 100% on all projects inspired me during difficult periods and I will be forever grateful.

Lastly, I would like to thank the Biomedical Engineering Department for the tremendous support that I have received and to acknowledge the various sources that have funded my graduate education. Those sources include the GEM Consortium, Alliance for Graduate Education in the Professoriate (AGEP), and the National Institute for Deafness and Other Communications Disorders (NIDCD).

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Abstract

Characterization of Multicompartmental Microparticles for Cochlear Drug Delivery

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Cochlear implants (CIs) are the preferred treatment for patients with moderate to profound sensorineural hearing loss. However, increasing numbers of CI recipients have remaining hearing that must be protected from insertion trauma. Local delivery of therapeutics could protect these initially viable inner ear sensory cells. This dissertation characterizes multicompartmental microparticles and their use for inner ear drug delivery.

The first part of this dissertation involved the determination of preferred design and infusion parameters for local drug delivery in the cochlea using multicompartmental microparticles. Parameters were classified as preferred if their modulation increased visualization of microparticles within cochlear tissues. Preferred parameters were associated with particle design (fluorescence intensity), particle infusion protocol (composition of delivery matrix), or tissue processing and post-harvesting (non-vascular tissue fixation).

In the next part of the dissertation *in vivo* microparticle persistence, distribution, and impact on cochlear function and histopathology were assessed in a guinea pig animal

model. Imaging of cochlear cross sections demonstrated the presence of microparticles for at least 7 days post infusion. Functional analysis with auditory brainstem response and histopathological analysis demonstrated that an infusion of non-drug loaded particles could be delivered with limited negative impact on hearing and cell viability and did not induce an immune response.

The final part of the dissertation evaluated *in vitro* and *in vivo* pharmaceutical release from multicompartmental microparticles. Incorporation and sequestration of Piribedil, an anti-excitotoxic agent, within a particular microparticle compartment was confirmed. *In vitro* microparticle assessment demonstrated sustained Piribedil release on the order of weeks which is suitable for intracochlear drug delivery. Analysis of perilymph obtained 7 days after *in vivo* infusion of Piribedil-loaded particles identified the pharmaceutical at detectable levels. For the first time, release of therapeutic levels of a pharmaceutical from an intracochlear particle system was demonstrated. This work provides the first evaluation of multi-release particles for local drug release in the cochlea for both *in vitro* and *in vivo* environments; defines the challenges to efficacious use of these drug carriers for modifying inner ear function; and identifies a research pathway that may enable clinical translation of these drug carriers for treatment of inner ear pathologies.

Chapter 1
Introduction: Cochlear Trauma and Drug Delivery

1.1. Impact of Hearing Loss

Approximately 36 million Americans suffer from hearing impairment (Donahue, 2010). These impairments influence many facets of an individual's life from social interactions to employment opportunities (Mohr et al., 2000). While those unaffected by hearing loss may take the use of this sense for granted, the process of hearing is multifaceted and complex. The ear is comprised of three distinct parts: the outer, middle, and inner ears. In normal hearing processes (**Figure 1-1**), sound waves enter the outer ear and then induce bone vibrations in the middle ear. The propagation of vibrations in the middle ear stimulate fluid movement in the inner ear (cochlea) which in turn stimulates hair cell vibrations that result in electrical signals that subsequently are sent to the auditory nerve cells. These nerve cells carry the electrical signal from the cochlea to the brain, eventually to the auditory cortex, which enables the initial sound to be perceived (Waltzman, 2006). However in individuals with hearing impairments, at least one part of this hearing cascade is disrupted. The causes of hearing loss are varied ranging from autoimmune diseases and infection to physical injury and environmental stress, including noise-induced trauma (Poe & Pyykkö, 2011; Swan et al., 2008). As a result, a wide variety of treatment options are available to hearing impaired patients depending on the severity of their impairment with options ranging from hearing aids to cochlear implants. Before understanding how these options work, it is important to become familiar with key components of the inner ear as seen in **Figure 1-2**. The cochlea is a spiral-shaped

organ with membranous and bony components. In humans, there are 2.5 turns and in guinea pigs, a common auditory research model, there are 4 turns. There are three chambers in the cochlea: the scala tympani, scala media, and scala vestibuli. Each of the respective chambers of the cochlea is filled with fluid. The fluid in the scala tympani and scala vestibuli is called perilymph and consists of a relatively high concentration of sodium ions. Conversely, endolymph, the fluid in the scala media, has a relatively high concentration of potassium ions. There is communication between the scala tympani and scala vestibuli particularly at the apex (the top of cochlea) in an area called the helicotrema. The chambers are separated by the basilar membrane, composed of mesothelial cells, and Reissner's membrane, composed of epithelial cells (Raphael & Altschuler, 2003).

The organ of Corti, the primary hearing organ, is located in the scala media. Hair cells, the sensory transduction cells of the cochlea, reside in the organ of Corti and thereby the scala media. There are three rows of outer hair cells and one row of inner hair cells. Outer hair cells aid in amplification of the sound wave providing sensitivity and selectivity. Inner hair cells act as mechanotransducers converting the mechanical energy imparted by the fluid movement to initiate depolarization of spiral ganglion neurons (SGNs), a type of auditory nerve cell. The depolarization of these cells results in the generation of an electrical signal that can be transmitted to the brain to enable the perception of sound (Raphael & Altschuler, 2003). The cochlea has two membrane covered openings to the middle ear, the oval window and the round window, which serve important functions. The oval window formed largely by the foot plate of the stapes, covers the opening of the scala vestibuli and is the site of initiation of the fluid wave in the cochlea since it comes

into direct contact with the bones of the middle ear. The round window is located at the base of the scala tympani, at the other end of the perilymphatic canal. The round window membrane functions to relieve pressure in the scala tympani by moving in response to a pressure gradient created by fluid movement.

The cochlea is tonotopic meaning that there is a correlation between function and cochlear location; specifically different portions of the cochlea are more readily activated by different frequencies of stimulation. The basal portion of the cochlea is most responsive to high frequency sounds while the apex is most responsive to low frequency (low pitch) sounds, **Figure 1-3**. Both the basilar membrane and the outer hair cells contribute to the tonotopic nature of the cochlea. The basilar membrane vibrates in response to pressure differences across the scala media, however the frequency of the sound source determines the location of maximal displacement of the membrane. From the base of the cochlea to the apex the basilar membrane thickens and widens while the length of outer hair cells increases. As a whole the structure from base to apex varies systematically in mass and stiffness, with the greatest stiffness and least mass at the base. Thus the resonance varies continuously from base to apex, with the base most responsive to high frequencies. Hearing aids are external devices that augment hearing by simply amplifying incoming sounds before they enter the ear canal at which point the normal hearing cascade occurs. Since hearing aids require functioning sensory and neural cells, they are not the preferred option for individuals with substantial damage to these cell populations. Cochlear implants work by bypassing sensory cells and providing direct electrical stimulation from their placement in the scala tympani to the auditory nerve cells which resides right above the implant as seen in **Figure 1-4** (Richards & Wise 2008).

Generally implants consist of a microphone, auditory processor, implant coil, and electrode array as seen in **Figure 1-5**. Cochlear implants are often inserted through the round window membrane or via a cochleostomy into the scala tympani. External sounds are amplified by the microphone and auditory processor before transmission to the implant coil. The implant coil propagates an encoded electrical signal to the electrode array which is used to stimulate nearby auditory neurons. While hearing aids and cochlear implants can provide great benefit, they are far from perfect and in need of improvement, leading researchers and clinicians to strive to combine technologies and techniques to enable patients to experience a therapeutic benefit greater than the respective interventions could provide separately. A major strategy for the future will include the combination of biotechnological (devices) and pharmaceutical treatment.

1.2. Causes (Sensorineural) & Hair Cell Loss and Intervention Strategies to Preserve Hair Cells

There are two primary types of hearing loss, conductive and sensorineural. Conductive hearing loss is characterized by damage to the outer and/or middle ear while sensorineural hearing loss is characterized by damage to the inner ear. Common treatment for conductive hearing loss is surgery and/or hearing aids. Treatment for sensorineural is more complex and the current treatment standard for moderate to severe pathology is the cochlear implant (CI). Cochlear implantation can result in hair cell loss in addition to the loss of peripheral processes of the auditory nerve as a result of physical trauma induced via device insertion. Eshraghi et al. demonstrated that even soft insertions with electrodes designed to minimize damage to the cochlea are likely to induce injury at the molecular level that cannot be observed macroscopically (A. A. Eshraghi, and T. R.

van de Water, 2006). Cochlear insult is believed to induce bursting of the peripheral process and the formation of reactive oxygen species (ROS). Therefore, the inhibition ROS formation via pharmaceuticals such as anti-oxidants appears to be a promising therapeutic route (Ohlemiller et al., 1999). The utility of this approach was evident in a study by Duan et al. (Duan, 2004). In this work, sensorineural hearing loss was induced in rats via high levels of impulse noise (similar to that that may be encountered with the use of firearms or as a result of exposure to industrial equipment). Prior to and after noise exposure, rats were treated with doses of the antioxidant, N-L-acetylcysteine (NAC), to assess the ability of this treatment to protect the cochlea from oxidative damage. Rats which did not receive antioxidant treatment served as the control and auditory-evoked brainstem responses (eABRs) were used to evaluate hearing loss. Rats which received a total of three NAC doses (one given an hour before noise insult, one given immediately following noise insult, and one given three hours after insult) were found to exhibit decreased hearing loss as compared to the controls thereby suggesting the protective value of antioxidant therapy on the cochlea.

Additionally, tumor necrosis factor α, a known mediator of cochlear inflammation, is released post-injury and as a result of this release induces hair cell loss (Satoh, 2002). In this instance, the use of a steroid such as dexamethasone (Dex) has shown promise for the prevention of hair cell loss as a result of noise-induced and physical trauma (Bin Wang, 2013; James DP & SJ, 2008). For example, direct infusion of dexamethasone into the guinea pig cochlea prior to noise-induced trauma was shown to have a protective effect (Takemura et al., 2004). Specifically, guinea pigs were treated with various doses of Dex (ranging from 1-1000 ng/ml) or artificial perilymph (AP) as a control for four days and

then exposed to a 120 dB noise at a frequency of 4kHz for 24 hours. As compared to those that received AP, a dose-dependent response in the attenuation of hair cell damage was noted. In another guinea pig animal model, the ability of corticosteroids to attenuate hair cell loss after cochlear electrode implant trauma was evaluated (A. a. Eshraghi et al., 2007). The authors inserted a cochlear electrode into guinea pigs who then did not receive further treatment or who received an infusion of either AP or Dex respectively for eight days after implantation. The contralateral ear of each guinea pig tested served as an internal control and the hearing loss of the implanted ear was compared to that of the control ear. ABR results show that by day 30 ears in animals receiving Dex infusions after electrode insertion did not demonstrate significant hearing loss as compared to their contralateral controls irrespective of the frequency tested. Moreover significant hearing loss was noted in animals who received infusions of AP or no treatment at all thus indicating the potential of this strategy in attenuating hearing loss caused by cochlear implantation.

1.3. Importance of IHC-AN Connections

One mechanism implicated in neural cell death post implantation is excitotoxicity in postsynaptic afferent fibers of the auditory nerve. There are two modes of excitotoxicity in the cochlea: 1) deterioration of the inner hair cell-auditory nerve (IHC-AN) connection induced by inner hair cell apoptosis or 2) bursting of auditory nerve processes in response to excess glutamate release from the IHC onto the IHC-AN synapse (A. A. Eshraghi, and T. R. van de Water, 2006). One of the dominant approaches in the field has been to attenuate excitotoxicity by preserving IHCs and subsequently the IHC-AN connections and neurons (Le Prell, 2007). Noise studies by Puel, demonstrated successful

reconnection of neuronal processes with IHCs following insult as long as the IHCs were maintained, leading to the assumption that the presence of IHCs indicated the presence of functional IHC-AN connections. However recent studies by Kujawa indicate that reconnection may not be as efficient as initially thought with significant losses of IHC-AN connections observed even when IHCs are preserved (Harrison, 2011; 2009).

1.4. Pharmaceutical Intervention for Cochlear Pathologies: Focus on Anti-Excitotoxicity Therapy

Anti-oxidants, corticoids/anti-inflammatory agents, and anti-excitotoxic agents are all classes of pharmaceuticals that have been investigated as interventions for ameliorating inner ear pathologies. Corticosteroids are used to reduce inflammation (Kitajiri, 2002). The primary function of anti-oxidant administration to the cochlea is to enable these agents to be used as scavengers of reactive oxygen species (ROS) which are toxic to the cellular and sub-cellular structures of the cochlea, particularly hair cells. Anti-oxidants also directly inhibit the apoptotic pathways activated by the presence of reactive oxygen species. Anti-excitotoxic agents are delivered to the cochlea to promote survival of neural cells and inner hair cell auditory nerve (IHC-AN) synapses. These drugs inhibit the sensitivity of nerve receptors to the excessive neurotransmitter release that occurs in pathological conditions following trauma. Glutamate is the primary neurotransmitter that is overreleased and in the absence of inhibition, auditory neurons are overstimulated causing calcium channels to be open excessively leading to an excessive influx calcium. This disproportionate calcium influx is accompanied by an influx of water into the nerve leading bursting of peripheral processes which results in deafferentation of the neuron and ultimately cell death. Therefore, a subclass of anti-excitotoxic agents of interest

would be those that act to decrease responsiveness to or the release of glutamate. Further rationale for selecting glutamate inhibitors is that it acts as a non-competitive antagonist and thereby would not interfere with the hearing cascade under normal conditions. Previous studies using competitive inhibitors have demonstrated decreased excitotoxicity in the auditory nerve in the presence of the aforementioned inhibitors albeit in the presence of serious side effects such as hearing loss, psychosis, and vomiting (Jager, 2000; Kitajiri, 2002). Potential non-competitive anti-excitotoxic agents are Memantine and Piribedil. Memantine and Piribedil are both neurotrophic agents that have previously demonstrated potential to inhibit excitotoxicity in the ear. Piribedil, a dopamine agonist, acts chemically to inhibit the inner hair cell auditory nerve (IHC-AN) synapse of afferent dendrites while Memantine, a N-Methyl-D-aspartic acid (NMDA) receptor antagonist physically prevents binding of stimulatory glutamate to prevent synaptic activity (d'Aldin, 1995; Oestreicher, 1998). A previous study with Piribedil utilizing intracochlear delivery of free drug prior to noise exposure found that treatment resulted in decreased excitotoxicity of the auditory nerve as evidenced by the lack of swelling and bursting of the peripheral processes (Oestreicher, 1998). In addition, there was attenuation of the number of IHC-AN connections lost after noise exposure in the presence of noise (d'Aldin, 1995). Further d'Aldin and colleagues demonstrated that this infusion of free drug reduced the compound action potential (CAP) amplitude after noise exposure without impacting the cochlear microphonic (hair cell) potential, which indicates that the treatment provided improved IHC-AN connection survival following insult without significantly impacting hair cell survival (1995).

1.5. Challenges of Drug Delivery to the Cochlea

While pharmaceuticals have the potential to positively impact cochlear health, they must first reach the cochlea in order to do so. Delivering drugs to the cochlea is complex because numerous challenges and obstacles impede therapeutic delivery to the cochlea. One of the most significant challenges is bypassing the blood-labyrinth barrier (BLB) (Swan et al., 2008). Similar in function to the blood-brain barrier, the BLB serve as a barrier between substances in general circulation and inner ear fluids thus limiting the diffusion of drugs and molecules from the vessels supplying the cochlea to the cochlear compartment. The efficacy of this barrier is derived from the tight junctions of which the BLB is comprised as these junctions help maintain a distinct chemical composition of the inner ear that differs from that which is found in the blood circulation. Another cochlear characteristic that imposes challenges on cochlear drug delivery is the limited diffusion of agents within the cochlea. There is negligible fluid flow in the cochlea; therefore drug dispersion is primarily by passive diffusion (Alec N. Salt & Plontke, 2009). This can result in a concentration gradient along the cochlea with cochlear locations and fluids distal from the infusion site receiving small amounts or essentially none of the intended therapeutic.

Though there are inherent cochlear characteristics that inhibit drug delivery, the drugs themselves can be limiting factors. In order to be effective, a drug must persist *in vivo* long enough to reach its target destination and/or deliver therapy for a defined time period (Chen, 2010). Therefore, the drug half-life, or the time it takes for half of a given dose to be eliminated from the body, is an important consideration. Therapeutic efficacy in cochlear delivery would be enhanced if the half-life of potential therapeutics were increased. Moreover, the drugs utilized in cochlear drug delivery may have unwanted

side effects that may hinder their use. Thus it would be advantageous to limit the number and/or severity of a drug's side effects. The carrier used to deliver the drug also plays an important role in drug delivery particularly as it relates to immune response. The carrier should be designed such that it is unlikely to elicit a significant immune response. Should a significant immune response occur, immune cells such as macrophages may uptake the drug carrier, eliminating it from the cochlea which prevents efficacious drug delivery.

1.6. Drug Delivery (Systemic vs. Local)

The mode of delivery is also crucial to evaluating the efficacy of therapeutic approaches. Pharmaceuticals have been delivered to the cochlea by both local and systemic routes (Le Prell, 2007; Yamagata, 2004). Early on, systemic delivery was the primary mode of drug delivery to the cochlea and drugs could be administered either orally or intravenously (Zou et al., 2010). However, there are numerous drawbacks that inhibit the effectiveness of systemic delivery. One of the most prominent limitations of systemic delivery is that therapeutic levels of the drug may never reach the cochlea. This can occur for a myriad of reasons including the blood-labyrinth barrier, clearance of the drug from systemic circulation, and drug release from a carrier prior to arrival at the site of interest (Alec N. Salt & Plontke, 2009). Furthermore, systemic delivery is more likely to lead to unwanted side effects as there is increased opportunity for the drug to act on other systems.

Local delivery of therapeutics to the inner ear is preferred in order to bypass the blood-labyrinth barrier and enable drug concentrations within the cochlea to reach the levels required to provide therapeutic efficacy while minimizing side effects (Hendricks et al., 2008; Alec N. Salt & Plontke, 2005). Both passive and active means may be used

to locally deliver drugs to the inner ear. These methods may be further divided into two categories of delivery: intratympanic and intracochlear (Rivera T et al., 2012). Intratympanic delivery involves the placement of pharmaceuticals into the middle ear and relies on diffusion across the round window membrane for agents to reach the inner ear while intracochlear delivery is the direct placement of drugs into the inner ear fluids. Since cochlear implantations are intracochlear surgical procedures, the access provided to cochlear fluid compartments presents a window of opportunity for local delivery of therapeutic agents with explicit clinical applicability.

The composition of the drug delivery vehicle is important to its intended application and the use of polymers is attractive since these materials enable tailoring of properties such as elasticity, hydrophillicity/hydrophobicity, biocompatibility, degradation, and cell targeting through the combination of various monomers or surface modification. Additionally, a broad range of polymer materials are utilized in cochlear drug delivery including both natural and synthetic materials (Anderson, 2009; Horie et al., 2010; Noushi, 2005; Alec N. Salt et al., 2011; Shayanne A. Lajud & Samudra Sanyal, 2013). Intratympanic delivery which exploits the permeability of the round window membrane (RWM) is a common delivery mechanism when delivery from biodegradable hydrogels is desired. These hydrogels are loaded with therapeutics and placed directly onto the RWM (Tsuyoshi Endo, 2005). For example, porcine type 1 collagen was crosslinked and loaded with a protein known to promote the survival of spiral ganglion neurons (SGNs) (brain derived neutrotrophic factor (BDNF)) and then placed onto the RWM. The gel delivered protein to the inner ear for 7 days and resulted in the enhanced survival of SGNs. Other biodegradable hydrogels were placed on the RWM to facilitate

pharmaceutical delivery to the cochlea include natural polymers such as chitosan-glycerophosphate, gelatin and synthetic polymers like siloxane-based polymers (Arnold et al., 2005; Lee et al., 2007; Paulson DP, 2008). The round window microcatheter is another method which exploits intratympanic delivery to the inner ear (S. K. Plontke et al., 2009). Here, a sustained release microcatheter was placed in front of the RWM via a typanomeatal flap and was attached to an electronic pump which was used to deliver dexamethasone, the therapeutic of interest. However, the use of intratympanic delivery was hindered in that it led to uncertainty as to the amount of drug that reaches the cochlea due to drug losses through the Eustachian tube in the middle ear and variations in the thickness and composition of the RWM itself (Pararas et al., 2012).

Intracochlear delivery circumvents this concern by directly accessing the cochlea via surgical intervention. Several microfluidic technologies have been exploited in intracochlear drug delivery including osmotic pumps, reciprocating microfluidic systems, and incorporation of microfluidic channels on the cochlear implant itself (Borkholder, 2008). For example, mini-osmotic pumps were used in conjunction with microcannulas to facilitate sustained delivery of brain derived neurotrophic factor (BDNF) to the rat cochlea (McGuinness & Shepherd, 2005). In particular, rats were profoundly deafened and then chronically given BDNF or artificial perilymph (AP) for a 28 day period. At the end of this 28 day period, animals that received chronic doses of BDNF had a spiral ganglion neuron (SGN) population that was similar in histological appearance and neural density as compared to control animals that had not been deafened. This was in stark contrast with the animals which received doses of AP as the histological appearance and neural density of these animals were comparable to that of animals which had received no

therapeutic following the deafening process. Direct fabrication of microchannels onto the cochlear implant itself may be used to facilitate drug delivery to the cochlea. Shepherd et al. developed an electrode array that could chronically deliver pharmacological agents to the scala tympani (Shepherd & Xu, 2002). Here, electrode arrays were connected to mini-osmotic pumps and implanted in guinea pigs. Sustained delivery of the pharmaceutical agent of interest from the array was maintained for a 28 day period while stimulating the auditory nerve suggesting this method as a promising means of cochlear drug delivery.

Apart from microfluidics, several other strategies are being developed for local drug delivery to the cochlea including viral vectors and micro/nanoparticles. Viral vectors, or gene therapy has been employed in the delivery of molecular therapeutics to the cochlea (Husseman & Raphael, 2009; Konishi, 2008). Recent studies have focused on the use of adenovirus (Ad) and adeno-associated viruses (AAV) as cochlear therapeutics (Staecker et al., 2011). For example, a multichannel electrode was inserted into a deafened guinea pig that was simultaneously given a single inoculation adenovirus construct that contained a BDNF gene insert (Chikar et al., 2008). Electrophysiological and psychophysical data was collected over an 80 day time period following cochlear implantation. Results indicated that auditory nerve survival was enhanced in animals that received the adenovirus in conjunction with electrical stimulation as compared to those that received electrical stimulation alone. Though promising, this approach is not without its concerns. Most specifically, the use of a virus poses significant toxicity concerns related to immunogenicity and some of the vectors can be difficult to generate (Staecker et al., 2004). Furthermore, lack of cell targeting is an issue because vectors are randomly dispersed within the cochlea limiting the number of genes that can be efficiently

delivered to auditory neurons. Genes must be efficiently delivered to the intended target site in order to have the desired therapeutic impact.

1.7. Particles as a Means of Facilitating Drug Delivery to the Cochlea

There are multiple ways to locally deliver therapeutics to the cochlea. The first of these is direct injection of the therapeutic into the cochlear space. However, the half-life of drugs directly injected into the cochlea is relatively short (on the order of minutes or hours) which limits the utility of this approach. Furthermore, when a drug is injected it is likely to accumulate at the site of injection rather than being distributed along the cochlear spiral. The preferred method is to use a drug delivery vehicle such as micro- or nanocarrier. This method is favored because loading a drug into a micro- or nanocarrier enables the sustained release of drug over time (on the order of days or weeks) which affords greater distribution and accumulation of the drug along the cochlear spiral therefore providing higher therapeutic benefit. A summary of the local drug delivery strategies investigated in the cochlea may be seen in **Table 1.1**.

Amongst drug delivery vehicles, polymeric micro- and nanoparticles have gained prominence. Several particle types have been utilized to facilitate therapeutic delivery to the inner ear including hydroxyapatite nanoparticles, silica nanoparticles, and polymeric nanoparticles (Chen, 2010). Of these, poly-lactic glycolic acid (PLGA) polymeric particles are particularly promising as they can be designed to meet many of the desired criteria for a drug delivery vehicle. These criteria include biocompatibility (PLGA is an FDA approved material), controlled drug release (by controlling the degradation characteristics of the polymer(s) utilized), tailored size and shape such that particles persist in the cochlea (resulting from control of fabrication process parameters), loading

of multiple pharmaceuticals into a single particle (as a result of particle compartmentalization), and potential for targeted delivery (via particle surface functionalization).

1.8 Objectives of This Work

This dissertation research focuses on the *in vitro* and *in vivo* characterization of multicompartmental microparticles for local delivery (via microparticle infusion) of therapeutic agents to the cochlea. The method of particle fabrication, electrohydrodynamic co-jetting, as well as the composition of the microparticles (compartments, materials, and incorporated therapeutics) were all selected to address several of the aforementioned challenges in the field. Within the broad framework of cochlear drug delivery, this work aims to:

1. Define microparticle and infusion parameters as well as post harvesting techniques required to enhance post infusion assessment of *in vivo* particle presence.

Hypothesis 1: Microparticles can be visualized in cochlear tissue. This hypothesis was tested through the use of confocal laser scanning microscopy to analyze cross-sections of cochleae infused with fluorescently labeled microparticles in *ex vivo* and *in vivo* guinea pig models.

Hypothesis 2: The composition of the particle delivery background matrix will influence the delivery of particles to the cochlea. This hypothesis was evaluated via observation of *in vitro* particle settling behavior and particle presence in cochlear cross sections following *in vivo* particle infusion.

Hypothesis 3: Particle porosity will impact the flow and distribution of particles following particle infusion. This hypothesis was assessed by quantifying distribution of particles of different porosities along a cochlear-like microchannel following *in vitro*

infusion. The ability to induce increased distribution of porous particles under infusion flow could allow encapsulated agents to reach more regions of cells within the cochlea, thereby potentially improving patient outcomes.

2. Determine if microparticle infusion will impact functional and histopathological aspects of cochlear health.

Hypothesis 1: Microparticles will be able to persist in the cochlea following *in vivo* infusion. This hypothesis was investigated by using confocal laser scanning microscopy, image analysis, and stereological analysis to assess the distribution and number of fluorescently labeled microparticles at 1 and 7 days following *in vivo* infusion.

Hypothesis 2: Microparticle infusion *in vivo* will not inhibit the function and viability of cochlear cells. This hypothesis was evaluated through the use of pre and post-infusion auditory brainstem response (ABR) thresholds and comparison of white blood cell infiltration and hair cell survival in treated ears when compared to untreated ears at 7 days post infusion.

3. Determine the *in vitro* release profile of particles loaded with a neuroprotective agent following *in vivo* infusion microparticles.

Hypothesis 1: Microparticles will be able to incorporate and sequester a relevant agent within a particular compartment. This hypothesis was assessed through the use of confocal microscopy to visualize the agent within the microparticle and determine the locations within the microparticle where colocalization occurred between the fluorescence of the agent and the polymer dyes used within different compartments of the microparticle.

Hypothesis 2: Microparticles will be able to release the agent in a sustained and controlled manner *in vitro*. This hypothesis was tested by ultraviolet spectrophotometry to assess release from drug loaded particles into aqueous media at multiple timepoints.

Hypothesis 3: Microparticle release of the agent will be detectable in the perilymph of animals that received *in vivo* microparticle infusions. This hypothesis was evaluated by sampling perilymph from animals infused with drug loaded particles and analyzing the perilymph with liquid chromatography mass spectrometry (LC-MS).

1.9 Overview of the Dissertation

Chapter 2 identifies key experimental design parameters for the particle delivery system.

Chapter 3 determines important factors that influence microparticle persistence and distribution in the cochlea *in vivo* and evaluation of cochlear tissue tolerance of infused microparticles.

Chapter 4 evaluates microparticle drug delivery release *in vitro* and *in vivo*.

Chapter 5 concludes the dissertation and discusses future research directions based on the findings of this work.

1.10 FIGURES & TABLES

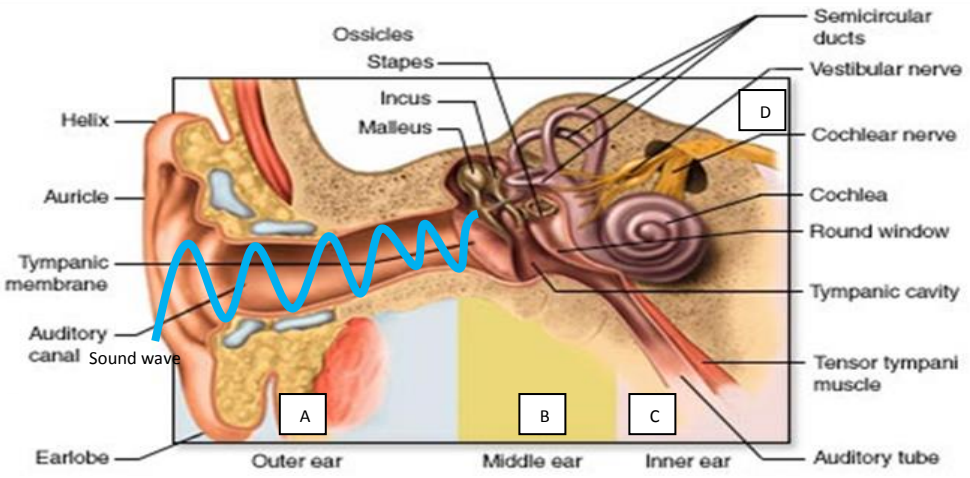


Figure 1-1. Illustration of sound processing in normal hearing. A) Sound wave enters the outer ear. B) Wave induces vibration of the bones in the middle ear. C) Bone vibration induces fluid movement within the cochlea (inner ear). D) Hair cells are stimulated by fluid movement and their vibrations cause electrical signals to be sent to the brain by underlying auditory nerve cells. Adapted from: (McGraw Hill)

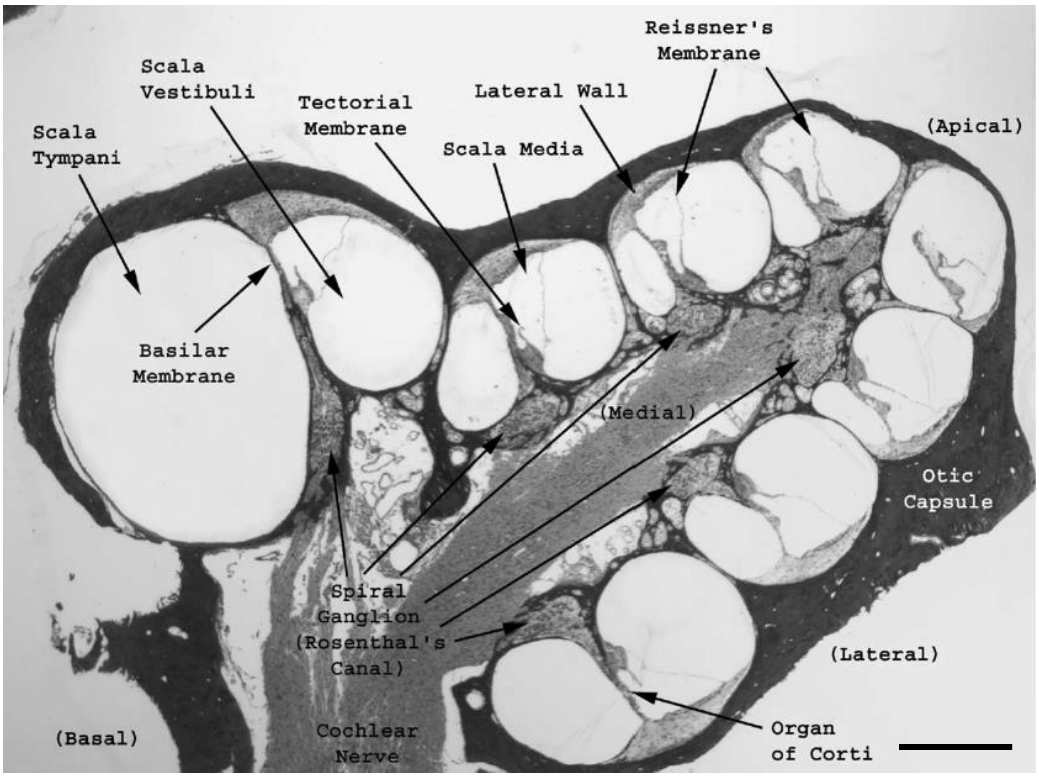


Figure 1-2. Low magnification view of the cross-section of the guinea pig cochlea displaying the respective cochlear chambers (Raphael & Altschuler, 2003). Scale bar is 0.5mm.

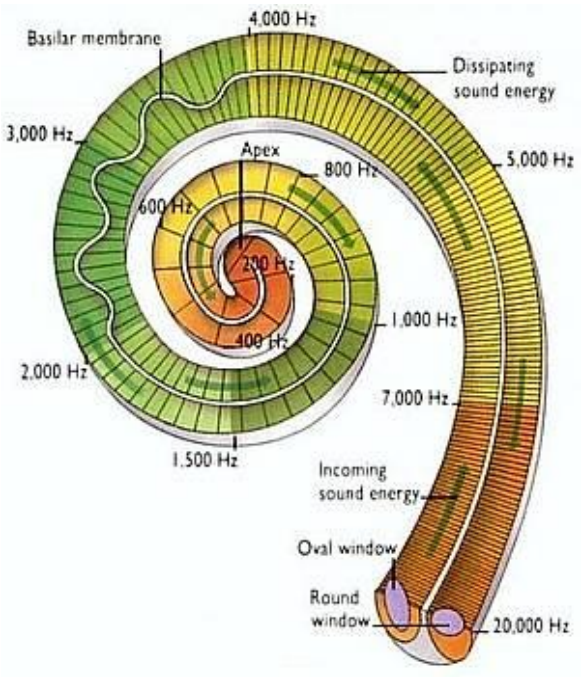


Figure 1-3. Tonotopic map of the cochlea. Frequency of sounds perceived decreases in a basal to apical gradient along the cochlear spiral (Shultz, 2012).

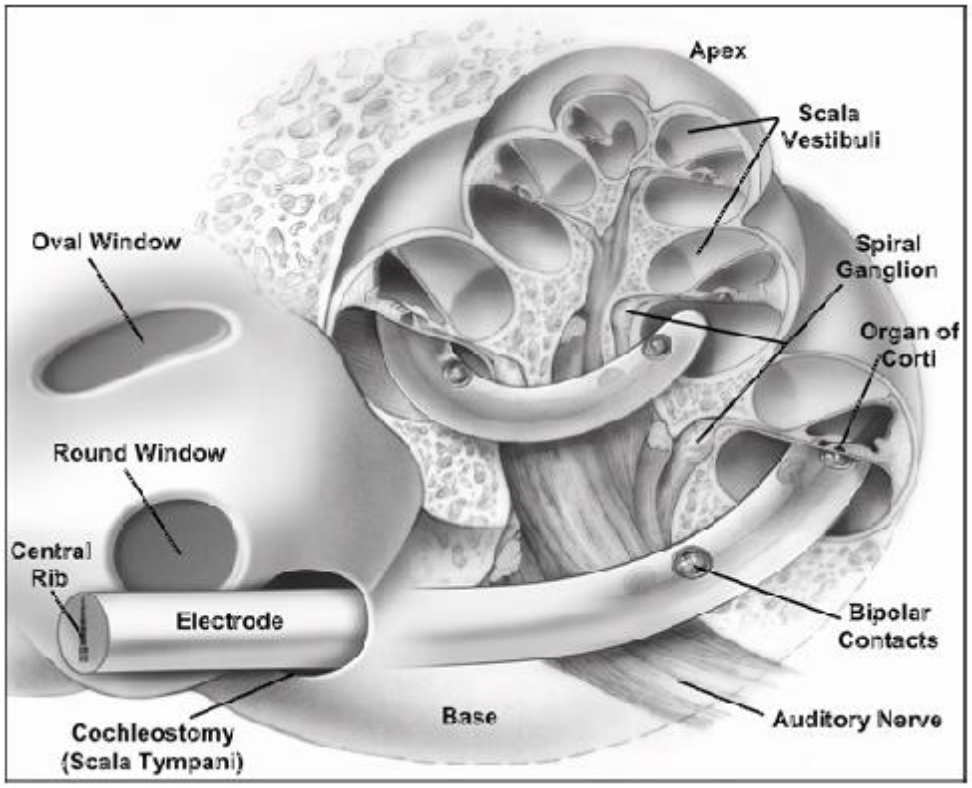


Figure 1-4. Cross-section of cochlea with implant denoting cochlear structures (Wilson & Dorman, 2008).

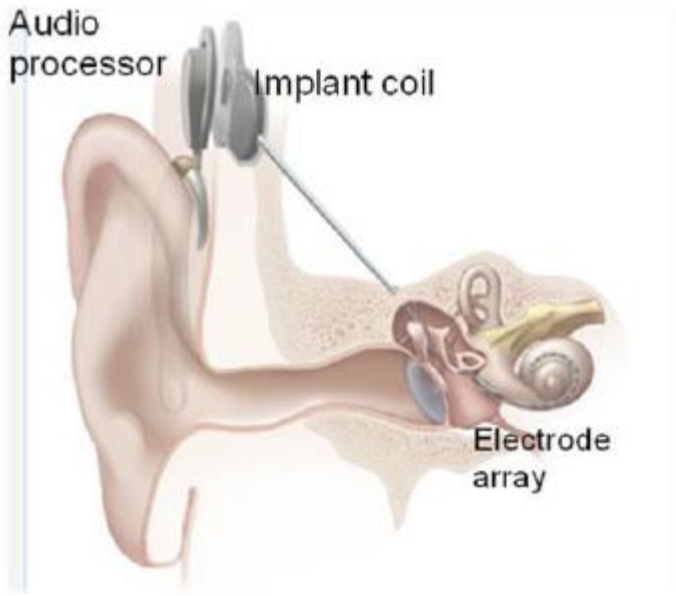


Figure 1-5. A cochlear implant consists of an external audio processor, implant coil, and electrode array. The audio processor turns sounds into signals that can be transmitted to the internal implant coil and converted to electrical signals. Electrical signals are sent to electrode array which directly stimulates nerve fibers in cochlea. Adapted from NIH ((NIDCD)).

Table 1-1. Potential Strategies for Cochlear Drug Delivery

Potential Inner Ear Carriers	Advantages	Limitations	Cochlear Distribution (turn)
Biodegradable Micro/nanoparticles	Biodegradable; Size; Multifunctional	Invasive	Variable
Mini Osmotic Pump	Continuous delivery	Must be refilled; Biofilm formation;	basal to apex
Viral vector	Induce regeneration	Induce immune response; toxicity	Variable
Gel/sponge	Ease of insertion	Extracochlear/Intratympanic application; drug incorporation by diffusion; Relies on RWM permeability	Basal

Chapter 2

Cochlear Drug Delivery Process Design Parameter Determination

Abstract

The use of pharmaceuticals to treat cochlear pathologies is a well-established practice. Traditionally clinicians have been limited to using agents that could either easily be injected into the middle ear or well-tolerated systemically. These requirements have restricted potential patient benefit from pharmaceutical intervention, especially those suffering from sensorineural hearing loss and requiring cochlear implantation because there are several agents that may improve outcomes in implantees that are not appropriate for delivery by either means. Intracochlear (local) drug delivery is an alternative delivery strategy capable of increasing the treatment options available to physicians and patients. The ideal carrier for local delivery would be functionalized and subsequently attached to the cochlear implant and/or cochlear cells, deliver multiple drugs with distinct pharmacokinetics, and sustain factor release over a defined period of time. Adequate distribution and ability to monitor distribution, e.g. via fluorescent probes incorporated into the carrier, would be important. To that end, fluorescent multicompartmental microparticles were evaluated to determine their utility for local drug delivery to the cochlea.

In this chapter, we characterize the feasibility of intrascalar delivery and detection of multicompartmental microparticles in cochlear tissues. Particles were fabricated from an

FDA approved biodegradable polymer and a polysaccharide via electrohydrodynamic co-jetting. Parameters impacting particle visualization and behavior within cochlear tissue were investigated using *ex-vivo*, *in vitro*, and *in vivo* models. In *ex-vivo* models guinea pig cochlea were harvested prior to infusion with microparticles (MPs) and in *in vivo* models cochlea were infused prior to harvesting. *In vitro* models were used to refine the fluorescent intensity of microparticles and particle solution composition and determine the impact of porosity on particle distribution under flow. Confocal laser scanning microscopy (CLSM) was used to confirm particle composition and multicompartmental nature. CLSM was also used to visualize particles in cochlear tissue. Scanning electron microscopy (SEM) was used to determine the impact of tissue processing agents on particle integrity. Important parameters to enable particle detection in cochlear tissue were identified and include: fluorescent intensity, viscosity of the particle solution, and fixation method. Refinement of the aforementioned parameters resulted in visualization of microparticles in cochlear tissue from both *ex-vivo* and *in vivo* models. Particle integrity as indicated by a smooth surface topology was maintained following exposure to tissue processing agents such as decalcification solution. Further confocal laser scanning microscopy confirmed that the fabricated particles had multiple distinct compartments. In addition, *in vitro* assessment demonstrated that particle porosity does not impact particle distribution under infusion flow conditions.

2.1 INTRODUCTION

2.1.1 Microparticle Design

It is known that the characteristics of microparticles (MPs) will either enhance or inhibit pharmaceutical release and degradation in their target environment over an extended period of time. Particle shape and size has previously been demonstrated to impact drug delivery, macrophage uptake, and cell uptake of particles (Champion & Mitragotri, 2009; Champion et al., 2008). Cell binding is size and shape limited because test articles must be sufficiently small as compared to a target cell. Cell uptake particles must have satisfactory surface area to volume (aspect) ratios to facilitate incorporation into intracellular spaces. In addition, size and global shape influence persistence of these vectors at the site of their initial attachment since these parameters determine the magnitude of shear forces generated against the particle and influence the likelihood that the vehicle will be dislodged (Patil et al., 2001). The size of target cells in the inner ear range from 8-20 microns in length, therefore a candidate microparticle with a diameter of approximately 8μm was selected as cell binding but not uptake was desired. Further, particles of this size are more resistant to phagocytosis by macrophages.

2.1.2 Materials Used in Cochlear Drug Delivery

Both synthetic and natural materials have been explored as carriers for pharmaceutical or growth factors delivered to the cochlea. Synthetic materials such as poly-l-lysine (PLL), polylactic-co-glycolic acid (PLGA), and poly(ε- caprolactone) (PCL) have been investigated as potential drug carriers to attenuate pathological conditions of the inner ear (Anderson, 2009; Chen, 2010; Parker et al., 2007). These materials are relatively inert; however, PLGA is the only biodegradable, FDA approved polymer to be studied. Polylactic and glycolic polymers are biodegradable and biocompatible because they

degrade *in vivo* to lactic and glycolic acid. These products are then naturally eliminated from the body via the Krebs cycle as carbon dioxide and water (Dunne et al., 2000). Furthermore, the polymer degradation is tunable because by changing the ratio of lactic to glycolic acid, the degradation rate (weeks to months) of the polymer may be modified to meet therapeutic needs. Previous work has been done with PLGA particles in the cochlea. The Ito group utilized PLGA nanoparticles (140-180 nm diameter) loaded with rhodamine dye to assess drug delivery to the cochlea by systemic and local delivery methods (Tamura et al., 2005). Particles were fabricated by an oil-in-water solvent diffusion method and administered systemically by intravenous injection. Two hours after systemic administration, 533.8±24.8 rhodamine nanoparticles were found in the liver while only 0.1±0.1 particles were found in the cochlea. Twenty-four hours following local application of these same nanoparticles to the round window membrane (RWM), particle presence, primarily in the basal turn, was observed. The number of particles, 28.8±4.5, observed in the cochlea 24 hours following RWM application was much greater than that observed following intravenous administration, although still small for therapeutic efficacy.

In follow-up work the Ito group utilized micron sized particles to locally deliver lidocaine, a pharmaceutical agent that has been shown to suppress tinnitus, to the cochlea (Horie et al., 2010). Microparticles (MPs) of two sizes, Lido-L (100±3.0 μm) and Lido-S (5±0.5μm), were fabricated using emulsification by the homogenization-solvent evaporation method. Evaluation of *in vitro* release profiles determined that the Lido-S particles released the majority of their payload in a burst release that was unsuitable for the sustained treatment required and no additional experiments were performed with this

particle. The Lido-L particles demonstrated a sustained release of lidocaine during *in vitro* study and were evaluated in guinea pigs *in vivo*. The greatest *in vivo* perilymphatic concentration of lidocaine achieved was 0.8µg/mL which is lower than the 10.8mg/mL fluid concentration required for tinnitus alleviation with local administration. In addition functional hearing deficits as measured by auditory brainstem response (ABR) were present at 7 days post-round window membrane placement of Lido-L particles. Thus limited drug accumulation and negative impact on cochlear function limits the utility of this approach moving forward.

Concerns about usage of a material that forms acidic, albeit natural byproducts, in the pH sensitive fluid environment of the cochlea may be addressed by modulation of particle characteristics such as size and porosity. For example, in *in vitro* dissolution studies with PLGA microparticles at various temperatures, including physiologic temperatures (37°C), found that large (greater than 50 µm) particles had a faster degradation rate than smaller particles (less than 20µm). (Dunne et al., 2000) The rationale for the observed phenomenon was that in the smaller particles (less than 20µm), degradation products formed within the particle diffused easily to the surface while in larger particles (greater than 50µm) the path to the surface of the particle was longer which caused slower diffusion of degradation products to the surface. During this reduced efflux, the prolonged presence of the degradation products induced an autocatalytic degradation of the remaining polymer material. Visual evidence of an acidic environment within degrading PLGA microspheres has been produced in the last several years (Fu et al., 2000). Through the use of confocal microscopy, the Langer group found that small (less than 15µm diameter microspheres) had no significant acidic area during

incubation in a neutral buffer solution (pH 7.4) and the average pH within the particles was maintained within 3% of the buffer solution. In contrast, pH for medium sized particles (up to 25μm) and large particles (up to 40μm), were only within 17% and 30% of the buffer solution pH respectively. By selecting a particle size of approximately 8μm for assessment of cochlear drug delivery, we are well below the size range of concern with respect to acid accumulation within particles. Furthermore by exploring the use of porous particles we could further protect against the development of acidic particle interiors. This reflects increased exchange between the particle and the incubation medium in which it is contained. As porosity is increased, ability to form extremely acidic microenvironments should decrease because degradation products would be able to escape more readily from the interior and the surrounding medium would have easier intraparticle access thereby preventing acidification of the core.

Examples of other synthetic materials investigated for cochlear drug delivery include nanoparticles consisting of a block co-polymer containing poly(ethylene glycol) block poly(ε- caprolactone) 1,1_-dioctadecyl-3,3,3_,3_-tetramethyl-indocarbocyanine perchlorate (DiI) and Labrafac1 WL 1349, an oil made of capric and caprylic acid triglycerides. Anderson et. al, investigated the delivery of nanoparticles consisting of a block co-polymer containing poly(ethylene glycol) block poly(ε- caprolactone) 1,1_-dioctadecyl-3,3,3_,3_-tetramethyl-indocarbocyanine perchlorate (DiI), a hydrophobic fluorescent probe (red). (2009) Poly-ethylene glycol (PEG) chains extending from the surface of the particle help to prevent particle aggregation in addition to reducing elimination of the microparticles from the cochlea due to immune response. This *in vitro* study evaluated the incorporation, distribution, and toxicity of amphiphilic block

copolymer nanoparticles (NPs) in spiral ganglion (SG) cell cultures. The authors found that DiI-loaded NPs were internalized into several cochlear cell types including guinea pig spiral ganglions, and SG glia/Schwann cells, neurons, in addition to human SG glia/Schwann cells. The particles were well tolerated in all the cell types studied as indicated by cell toxicity and viability assays. The particles studied had diameters of 83.5±17.6 nm. One of the limitations of this study was the use of poly(ε- caprolactone) which though biodegradable, has a degradation profile on the order of years, and therefore would persist indefinitely in the inner ear and may not enable release of a therapeutic dose of agents near the time of initial implantation.

Zou and colleagues fabricated liquid nanocapsules (LNC) under 50 nm in diameter that interacted with cochlear cells and tissues. This study demonstrated that LNCs can pass through the round window membrane (RWM), pass the cochlear partitions, and address inner ear cell populationsJing Zou (2008). LNCs were endorsed as potential vectors for drug delivery into the spiral ganglion cells, nerve fibers, hair cells, and spiral ligament. One of the limitations of this approach was that it depends on RWM permeability which varies from animal to animal.

An example of natural materials investigated for cochlea drug delivery is the alginate-heparin beads developed by (Noushi, 2005) to release NT-3, a neurotrophin demonstrated to promote the survival of auditory neurons after cochlear damage. The beads were created by ionic gelation and incubated with NT-3 overnight for a final NT-3 uptake of 98-99%. The authors were able to introduce 4-5 beads (0.5-1mm diameter) into the scala tympani via perforation of the round window and seal them into the cochlea using muscle fascia. At a dosing of 1.5 μg of NT-3 per bead, significant improvement in auditory nerve

survival was observed at one month and no significant inflammatory effect was found. The limitations of this study were potential variable drug loading and observed high burst release due to simple immersion of beads for NGF incorporation, and the large size of the beads relative to the scala (0.5 – 1.0mm) (Noushi, 2005). Recently, Buckiová et. al demonstrated the transport of fluorescently labeled liposomes and polymersomes through the round window membrane into the cochlea in a murine animal model. Particles were primarily found in the spiral ganglion neurons, but were also present in the lateral wall, organ of Corti, and spiral limbus (Buckiová et al., 2012). The toxicity of unloaded nanoparticles was assessed via auditory brainstem response and change in hearing threshold was observed after application of either type of nanoparticle. Nanoparticles were also loaded with a neurotoxic agent, disulfiram, to ascertain whether the particles were capable of delivering enough payload to induce a functional effect. Within two days of Disulfiram-loaded nanoparticle delivery, the number of spiral ganglion cells decreased significantly. Within two weeks, hearing threshold shifts of 20–35 dB were present as detected by auditory brainstem responses. The toxicity of particle payload appeared limited to the intended target of spiral ganglion cells because no changes in hair cell morphology or function were detected following delivery of disulfiram-loaded nanoparticles. In this study, morphology was observed via phalloidin staining and functionality was assessed by otoacoustic emission recordings. The materials used to fabricate the nanoparticles must be considered however, because the polymersomes are made of polyethylene glycol-/block/-poly(ε-caprolactone) and the liposomes also contain polyethylene glycol, which raises concerns about the long term impact of the degradation products on cochlear health. Further, the core-shell structure of both types of

nanoparticles restricts loading to one pharmaceutical per particle. Finally as with other drug delivery systems that rely on round window membrane permeability, the high inherent variability of this mode of transmission must be considered.

The use of nano and microtechnology for drug delivery often necessitates the dispersal of particles in an aqueous solution to facilitate delivery. The components of this solution are selected based on biological relevance or inertness in accordance with the intended application. The intended application will drive selection of solution components, however agents selected should be biologically relevant and inert. A homogeneous suspension may be made by vortexing, however, particle sedimentation may arise from differences in density between the dispersed phase (e.g. microparticles) and the continuous phase (e.g. artificial perilymph). Sedimentation occurs when the dispersed phase is more dense than the continuous phase. Thus sedimentation must be taken into consideration when designing a drug delivery vehicle for the cochlea.

2.1.3 Delivery schemes used for cochlear drug delivery

The maximum rate at which a solution may be pumped into the cochlea without causing damage to cellular structures is 1 µL/min (Alec N. Salt & Plontke, 2005). The volume of a guinea pig cochlea is approximately 10µL. The microliter volume of the cochlea means that even small leaks could result in the loss of significant amounts of both perilymph and infusion solution. Therefore delivery must include a ‘rest period” after infusion during which time the delivery cannula remains in place in the cochleostomy, to reduce efflux from the cochlea when the cannula is removed.

Recently, the Lahann lab at the University of Michigan (Lahann, 2011) developed a process, electrohydrodynamic co-jetting, that enables the fabrication of

multicompartmental microparticles. The microparticles are comprised of PLGA and PLGA/acetal dextran polymers. They can be fabricated in sizes ranging from submicrons to tens of microns and pharmaceuticals of interest may be incorporated directly into the polymer solution prior to jetting. A variety of shape configurations are also possible and the flexibility of platform in creating particles with selected characteristics is outlined in **Figure 2-1**. In this study we determined the process design parameters needed to enable the delivery and visualization of eight micron spherical microparticles within the cochlea. During the completion of this work the following hypotheses were addressed:

Hypothesis 1: Microparticles can be visualized in cochlear tissue. This hypothesis was evaluated using a guinea pig animal model. Following *ex-vivo* and *in vivo* infusion, cross-sections of cochleae infused with fluorescently labeled microparticles were assessed with confocal laser scanning microscopy (CLSM).

Hypothesis 2: The composition of the particle delivery background matrix will influence the delivery of particles to the cochlea. This hypothesis was investigated using *in vitro* and *in vivo* methods. Particle settling behavior was assessed *in vitro* and particle presence in cochlear cross sections was quantified following *in vivo* particle infusion.

Hypothesis 3: Particle porosity will impact the flow and distribution of particles following particle infusion. This hypothesis was explored via *in vitro* infusion of particles of various porosities along a cochlear-like microchannel. Following infusion, particle distribution was quantified to determine if there was correlation between particle porosity and distanced traveled. The ability to induce increased distribution of porous particles under infusion flow could allow encapsulated agents to reach more regions of cells within the cochlea, thereby potentially improving patient outcomes.

2.2 METHODS

2.2.1 Particle Fabrication

Particles used in the following study were made of poly-lactic- glycolic acid (PLGA) or poly-lactic-glycolic acid and dextran acetal(PLGA/dex). PLGA hybrid particles consisted of two types of PLGA, Purasorb 5004a (MW:44 kDa) and Purasorb 5002a (MW: 17 kDa). Both types of PLGA had a lactic to glycolic acid ratio of 1:1. PLGA/dex compartments contained 25% PLGA (MW: 44 kDa) and 75% Dextran Acetal. The polymer dye, poly[(m-phenylenevinylene)-alt-(2,5-dihexyloxy-p-phenylenevinylene (MEHPV) which was visualized with the blue channel during imaging was incorporated into both compartments of PLGA/dextran particles for all *in vivo* and *ex vivo* experiments. For compartmentalization studies, MEHPV was utilized in the PLGA/dextran compartment while poly[tris(2,5-bis(hexyloxy)-1,4-phenylenevinylene)-alt-(1,3-phenylenevinylene) (PTDPV) was used to delineate the PLGA only compartment (green channel during imaging). All particles were made by electrohydrodynamic co-jetting (Lahann, 2011), a process which involves a side-by-side capillary needle system containing polymer solutions and the application of an electric field to the system. The field stabilizes the interface between the solutions enabling the formation of an electrified polymer jet of particles with multiple distinct compartments. Some of the particles containing PLGA/dex compartments were also incubated in an acidic solution (pH=5) for 15 hours to facilitate pore formation on the surfaces of those compartments. Particles were then washed with PBS+1% Tween 20 five times to remove all acid, then filtered through a 10 μm filter. After filtration, particles were centrifuged down and the PBS removed. A schematic of the particle fabrication process may be seen in **Figure 2-2**. Prior to an experiment, a known mass of particles was suspended in artificial perilymph (AP;

145mMNaCl, 2.7mMKCl, 2.0 mM $MgSO_4$, 1.2 mM $CaCl_2$, 5.0 mM HEPES; pH = 7.35-7.40, osmolality = 285–300mOsm) or artificial perilymph with known percentage of bovine serum albumin (BSA) (*ex-vivo*) or guinea pig serum albumin (GPSA) (*in vivo*) to create a solution with a specific concentration (mg/mL) of particles for infusion. Due to the molecular weight of the PLGA/dextran used, microparticles fabricated with this co-polymer were labeled high density (HD) particles. Further, particles made completely of PLGA/dextran that were not exposed to the acidic incubation were labeled high density control (HDOC) particles. Two types of PLGA/dex particles were exposed to acidic incubation. The first type of particle was one that contained PLGA/dex in one compartment and subsequently had pores in only one compartment and therefore was referred to as a high density monoporous (HDO) microparticle. The second particle type contained PLGA/dex in two compartments and subsequently had pores in two compartments, high density bi-porous (HDOB) microparticle. A schematic of the PLGA hybrid and high density particles is seen in **Figure 2-3**. When used simultaneously in experiments, particles fluoresced in the blue/DAPI (HDOC), green/FITC (HDO), or red/Rhodamine (HDOB) channels respectively as seen in **Figure 2-4**.

2.2.2 Particle Characterization

Prior to infusion studies, scanning electron microscopy was used to visualize the surface morphology of the high density microparticles. Further, as visualization of the particles in tissue had not been attempted previously, the ability to identify particles in cochlear cryosections was assessed. This assessment utilized sections obtained from both *ex vivo* and *in vivo* infusions. The fluorescence intensity of 3 mg/ml blue & red/green dye in the microparticles versus commercially available polystyrene beads (PSB;

Fluoresbrite) was used to assess the efficacy for the infusion protocol. *In vitro*, bovine serum albumin was used to determine the concentration of guinea pig serum albumin that would be needed during *in vivo* infusions to reduce particle settling.

2.2.3 *In vivo* Infusion

For *in vivo* infusions, Hartley guinea pigs (Charles River Laboratory, Wilmington, MA) were anesthetized and the temporal bone was drilled to facilitate visualization of the cochlea. A fine pick was then used to create a small, less than 0.2mm, fenestra in the basal turn of the cochlea. A micro-cannula with a silastic ball was inserted into the scala tympani via the cochleostomy and cyanoacrylate was used to seal the cannula in place (Prieskorn & Miller, 2000). The microcannula was made from polyimide and the silastic ball was made from Sylgard (Dow Corning, Midland, MI).A syringe infusion pump (Harvard Apparatus**,** Holliston, Maine) was used to deliver particles to the scala tympani at a flow rate of 1µL/minute over 5 minutes. This rate was selected because it is the highest infusion rate that may be used in the cochlea without damaging cochlear cells and structures. In some experiments, commercially available polystyrene beads (PSB; Fluoresbrite) were used as a control to assess utility of the infusion protocol. Infusions were always performed on the left ear and the right ear was used as a contralateral control.

2.2.4 *Ex vivo* Infusion

In the *ex vivo* infusion model, guinea pig cochleae were dissected and decalcified prior to particle infusion. Specimens were stored at 4°C in 0.4% paraformaldehyde (PFA) until use. Upon use, a fine pick was used to create a small fenestra in the cochlear apex. A

syringe micropump was used to infuse 20 μL of 13mg/mL PSBs or 100mg/mL bi-colored MPs into the cochlea.

2.2.5 Cochlear Dissection

For *in vivo* specimens, guinea pigs were anesthetized and euthanized by cardiac perfusion with 0.1M phosphate buffer followed by 4% paraformaldehyde or by injection of sodium pentobarbital (FatalPlus; Vortech Pharmaceuticals, Dearborn, MI). In all cases, secondary euthanasia was performed by transecting the aorta and the ventricle. Animals were decapitated and skulls were opened along the midline to facilitate removal of the brain. The temporal bone which encases the cochlea was removed for both the left and right cochlea. The bulla of the bone was identified and bone chippers were used to remove the excess bone to facilitate visualization of the cochlea. The malleus, incus, and stapes bones were also removed. A sharp probe was used to create small fenestra in the apex to aid infiltration of fixative and specimens were fixed in 4% paraformaldehyde for at least 1-2 hours prior to preparation.

2.2.6 Decalcification and Cryoprotection of Specimens

Following fixation, cochleae were decalcified in a solution that is 2/3 formic acid (Immunocal; Decal Chemical Corporation,Tallman, NY) and 1/3 7% sucrose for 6-8 hours. Prior to freezing, specimens were placed in aluminum container and immersed in a 30% sucrose solution. Freezing was performed by placing the bottom of the container in contact with liquid nitrogen cooled 2-methyl-butane (Fisher Scientific, Pittsburgh, PA). Specimens were wrapped in parafilm and stored at -80°C until sectioning.

2.2.7 Cryostat Sectioning

Sections 14µm in thickness were collected from 0-2000µm in depth. The 0 depth was categorized as the first section where any portion of the cross section of the cochlear apical turn became visible. Serial sections were taken with every 2^{nd} and 3^{rd} section mounted on a microscope slide. Each slide contained 2 sections such that set of collected serial sections were contained on one slide. Slides were allowed to air dry for a minimum of one hour before being placed at -80°C until they could be mounted and imaged. Comparisons were made between slides from the same one hundred micron depth.

2.2.8 Confocal Laser Scanning Microscopy

An Olympus confocal microscope with Fluoview software was used to visualize and acquire images of the particles. Particles were excited at the following wavelengths as appropriate for visualization: fluorescein isothiocyanate (FITC), 4',6-diamidino-2-phenylindole (DAPI), and tetramethylrhodamineisothiocyanate (TRITC). The FITC channel was also used to aid the acquisition of differential interference contrast (DIC) images. Images were acquired with a resolution of 1024x1024 pixels. Specimens were mounted in Floumount (Electron Microscopy Sciences, Hatfield, PA) and coverslipped for imaging. The seal between the mounting medium and the coverslip was allowed to dry for a minimum of 2 hours prior to imaging.

2.2.9 Fabrication of Cochlear-like Microchannels

Silicone two part A:B (1:10; Dow Corporation) was mixed and poured on microscope slide and allowed to air dry. A mask in the shape of a rectangle with a consistent 1 mm diameter that spanned a length of 20mm was fabricated. At the end of the rectangular channel, a triangular outlet was added to provide an outlet for the infused microparticle solution. The coated microscope slide was placed on top of the mask and a scalpel was

used to cut away the silicone to create the channel. Infusions of 1mg/mL microparticles (0.33mg/mL of each particle type) in 30% bovine serum albumin (BSA) were made at 1µl/min. Microchannels were wetted with deionized water prior to infusion to facilitate distribution throughout the entire channel by decreasing the hydrophobicity of the surface and to more closely mimic the *in vivo* environment. Confocal images were taken immediately following infusion at the following points along the channel: 5, 10, 15, and 20mm. Images were collected at 20X magnification on an Olympus Confocal Microscope as described above.

2.2.10 Impact of Processing on Particle Integrity

Scanning electron microscopy (SEM) (Philips XL30 ESEM, high vacuum mode at 5kV) was used to image PLGA only particles that had been immersed in one of two decalcification solutions, either 5% EDTA or commercially available Immunocal. Decalification of cochleae immersed in 5% EDTA generally takes 2-3 weeks, however, decalcification in Immunocal requires less than 24 hours. Particles immersed in artificial perilymph served as a control. Particles were immersed in the solutions from 3 hours to 10 days. At the requisite timepoint, the respective solutions containing particles were centrifuged and excess fluid was aspirated. Particles were resuspended in distilled water and 10µL of the suspension was pipetted onto on graphite conductive coating disc (Electron microscopy sciences, Hatfield, PA) on top of a metal stub, in preparation for SEM. The suspensions were allowed to dry for 24 hours prior to sputter coating with gold (Polaron E5100 Sputter Coater) and imaging at approximately 1500X magnification.

2.2.11 Impact of Processing on Particle Persistence

Cochleae from infusion procedures were cryoprotected and sectioned as described previously in sections 2.2.6 and 2.2.7. Upon removal from storage at -80°C, sections were permitted to air dry and were either rinsed and mounted or mounted without rinsing to evaluate rinsing on particle counts and distribution.

2.2.12 Image J Analysis

Image J software (NIH, Bethesda, MD) and the cell counter plug-in were used to determine the number of particles of each type present in the cochlear tissue or a cochlear sized microchannel. When multiple particle types were present, the color of the fluorescence channel emitted from the particle was used to delineate one particle type from another. Each type of particle was given a different counter number (i.e. green/FITC particles were assigned counter number 2, blue/DAPI particles were assigned counter number 3, etc.) to enable counts of the various particle types to be performed.

2.3 RESULTS & DISCUSSION

2.3.1 Particle Characterization

Some characterization studies utilized PLGA only particles rather than the PLGA dextran particles. This paradigm provided good baselines (for intensity, particle integrity, etc.) because the same type of PLGA was used in both particle systems.

The fabrication of the high density PLGA/dextran acetal particles was confirmed by confocal laser scanning microscopy as seen in **Figure 2-5** whereby the PLGA/dextran compartment is indicated in the DAPI (blue) channel and PLGA only compartment is indicated in the FITC (green) channel. The surface morphology, including the porous nature of the monoporous and biporous high density particles when compared to the

control high density particles was visualized via scanning electron microscopy as seen in **Figure 2-6**. During initial *in vivo* experiments, particle settling was observed in the microsyringe during the time period after particle loading and flush of the cannula, but prior to the start of the rate controlled infusion of the particle solution into the cochlea. *In vitro* experiments, demonstrated that it took 15 minutes for particle settling to occur. It was determined that the viscosity of the microparticle/artificial perilymph mixture was too low and thereby enabled the particles to "fall-out" of solution and sit on the bottom of the microsyringe which reduced the number of particles delivered to the cochlea. In order to increase the viscosity of the background matrix used for microparticle delivery, albumin was added to the artificial perilymph. Albumin is a soluble protein naturally found in blood plasma and is biocompatible and biodegradable (Sayyed Abolghassem, 2003). Bovine serum albumin (BSA) was used in *in vitro* assessments and guinea pig serum albumin (GPSA) was used in *in vivo* assessments. GPSA was used in *in vivo* assessments to minimize the immune response. During the development of the albumin/artificial perilymph solution, a mismatch was discovered between the percentage of bovine serum albumin used to prevent settling in *in vitro* studies and that required to prevent the same phenomenon with the guinea pig serum albumin *in vivo*. GPSA was less dense than the BSA, therefore larger amounts of GPSA were required to obtain an albumin/perilymph solution of similar viscosity. For the purpose of these studies, desired viscosity was not directly measured rather it was evaluated as a function of particle settling time. The desired time was selected based on empirical observations and subsequent analysis of the amount of time that elapsed between particle loading into the microsyringe and infusion into the cochlea during surgery. The time interval was 30

minutes and 1% particles in a 30% BSA solution was determined to have the necessary viscosity to keep particles in suspension. To prevent settling in 1% microparticles for the same amount of time with GPSA, a 55% solution was required.

Visualization of the microparticles once they were infused into the cochlea was crucial to these studies. Therefore, concentration and fluorescence intensity experiments were conducted to identify optimal operating parameters for both factors. Microparticle concentrations evaluated were 20, 40, and 100 mg/mL respectively as seen in **Figure 2-7**. The 100 mg/mL concentration yielded approximately 1.4 million particles in 5μL. Previous studies showing functional efficacy of stem cells on the order of 1M, indicate that a target of 1.4M is appropriate (Parker et al., 2007). Initial work began with the 100mg/mL concentration, however it was ultimately decided to switch to 15mg/mL particle solutions because of particle aggregation and settling at the higher concentrations. At a concentration of 15 mg/mL, particle counts typically ranged from 325,000-350,000. If further reduction of particle aggregation became necessary, the particle surface could be modified to enable the attachment of polyethylene glycol (PEG) which reduces particle interactions due to steric hindrance (Fang et al., 2009).

Initially, it was difficult to discern the PLGA microparticles within infused cochlear tissue. Therefore, a commercial particle, Fluoresbrite, was identified and served as a comparison standard in intensity studies. The Fluoresbrite beads are made of polystyrene and were selected because of their intense fluorescence, good dispersion, and similar size, 6μm, to the microparticles, 8μm. Furthermore, there was outstanding spectral overlap between the microparticles (excitation at 488 nm) and the polystyrene beads (excitation at 491nm) which allowed the same laser to be used to visualize both

vehicles simultaneously for direct comparison of intensity. Additionally the commercial beads demonstrated outstanding visibility within cochlear tissue sections as seen in **Figure 2-8**. The amount of blue and red/green (B&R/G) dye needed for incorporation into dual color PLGA microparticles to provide good signal in the presence of the Fluoresbrite beads was also determined as seen in **Figure 2-9**.

In addition to providing a benchmark for fluorescence intensity, the non-biodegradable Fluoresbrite beads were also used to validate the chosen surgical infusion protocol to ensure that it enabled effective delivery of particles to the cochlea and that these particles could persist in the cochlea for at least 24 hours post-infusion. *Ex vivo* and *in vivo* guinea pig cochlea models were used to characterize the distribution of the microparticles and the control polystyrene beads within cochlear tissue. Large sections of intact cochlea and thin sections of cochlea were assessed. Both MPs and PSBs were detected *in vivo* and *ex vivo* (not shown) by 2 Photon CLSM in whole cochlear specimens after 24 hours as seen in **Figure 2-10**. Furthermore, microparticles and Flouresbrite polystyrene beads were both visible in cochlear cryosections of *ex vivo* and *in vivo* specimens at the 24 hour timepoint.

2.3.2 Processing Studies

Extensive post processing of mounted tissue was required for imaging and particle distribution assessment. Therefore it was important to assure that some of these steps were not inherently damaging to the particles because they may have made counting difficult and confounded results. Scanning electron microscopy (SEM) (Philips XL30 ESEM, high vacuum mode at 5kV) was used to assess PLGA particle morphology following immersion in the decalcification solutions used to prepare the cochleae for

cryosectioning. Particle response to two types of decalcification solutions, 5% (Ethylenediaminetetraacetic acid) EDTA and Immunocal, a commercial decalcification product were evaluated. Particle immersion in artificial perilymph served as a negative control. As expected, immersion in artificial perilymph did not change particle morphology. Furthermore, particle immersion for up to 24 hours in Immunocal and up to 10 days in 5% EDTA also appeared to allow maintenance of particle integrity as shown in **Figure 2-11**. The surface of particles remained fairly uniform and did not experience dramatic changes in size.

Once satisfied that the microparticles were sufficiently fluorescent to enable distinction from tissue and that the decalcification solutions did not significantly degrade the particles, the impact of slide processing steps were evaluated. Washing was needed for specimen slides when optimal cutting temperature (OCT) compound was used as the cryoembedding medium for infused cochleae as this material left a white film on the slide and within the tissue spaces. This film impeded detection and imaging of particles by confocal microscopy thereby necessitating washing. There was concern that wash steps may, in addition to eliminating the film, remove hydrophobic particles from the tissue specimens. Therefore to eliminate the need to wash slides, the cryoembedding medium was switched to a 70% sucrose solution which dries clear, and thereby does not require clearing with exogenous liquid agents to visualize microparticles within the cochlear tissue sections. To ascertain whether washing impacted the number of MPs observed in cochlear sections, particle counts were performed on cochlear cross sections exposed to wash steps as well as those mounted without washing. On average, 10% fewer particles were found in washed samples when compared to unwashed samples.

2.3.3 Perfusion/Non-perfusion

Initial *in vivo* studies did not demonstrate the presence of large numbers of particles within the cochlea following infusion. Post processing of cochlear tissues for cryosectioning and analysis involved an intracardiac perfusion of 4% paraformaldehyde to assure that the tissue was properly fixed. A poor cardiac perfusion revealed much greater numbers of particles than previously visualized, with association of particles with cochlear vasculature or blood clots. Future animals did not receive a vascular perfusion and confirmed a much larger number of particles present in the cochlea. Non-perfused cochleae had an average of 400% more particles than perfused specimens. Therefore, animals used in all subsequent experiments were not systemically perfused with paraformaldehyde to facilitate fixation.

2.3.4 Cochlear-like Microchannel

In addition to particle persistence, our goal was to assess particle distribution within the cochlea. In particular, we were to determine if different surface architectures of the microparticles impact their distribution within the cochlea. Our initial hypothesis was that increased porosity would respond to minute flow initiated by the micropump and facilitate infusion and distribution of the microparticles in the cochlea. To test this hypothesis, a small channel similar in width (1mm) and length (20mm) to the cochlea was fabricated and a microsyringe pump was used to infuse particles into the channel as seen in **Figure 2-12**. When used simultaneously in experiments, particles fluoresced in the blue/DAPI (high density control), green/FITC (high density monoporous), or red/TRITC (high density biporous) channels respectively. Distribution of HDOC, HDO, and HDOB particles within cochlear molds under experimental like conditions were assessed at four locations: 5mm, 10mm, 15mm, and 20mm from the infusion end of the

channel. Confocal images were taken at 20X magnification at all locations and the number of each type of particle present was evaluated using Image J. Though unexpected, the microparticle surface architecture did not seem to impact the distance the particles traveled as seen in **Figure 2-13**. This finding suggests that fluid flow produced by the syringe pump is too small to create the laminar flow needed to enhance distribution.

2.4 CONCLUSIONS

This work has demonstrated the feasibility of visualizing multicompartmental particles within the cochlea and its respective tissues. In order to accomplish this work, particle parameters such as size, fluorescence intensity, porosity, and concentration were refined. Further, infusion conditions such as microparticle solution composition and delivery rate were considered. Finally, the impact of post infusion processing including perfusion, decalcification, cryoprotection, and specimen rinsing were determined. Particle parameters, infusion conditions, and post processing were modified when necessary to facilitate increased visualization of microparticles in the cochlea, thereby enabling more rigorous assessment of particle presence and distribution.

2.5 FIGURES & TABLES

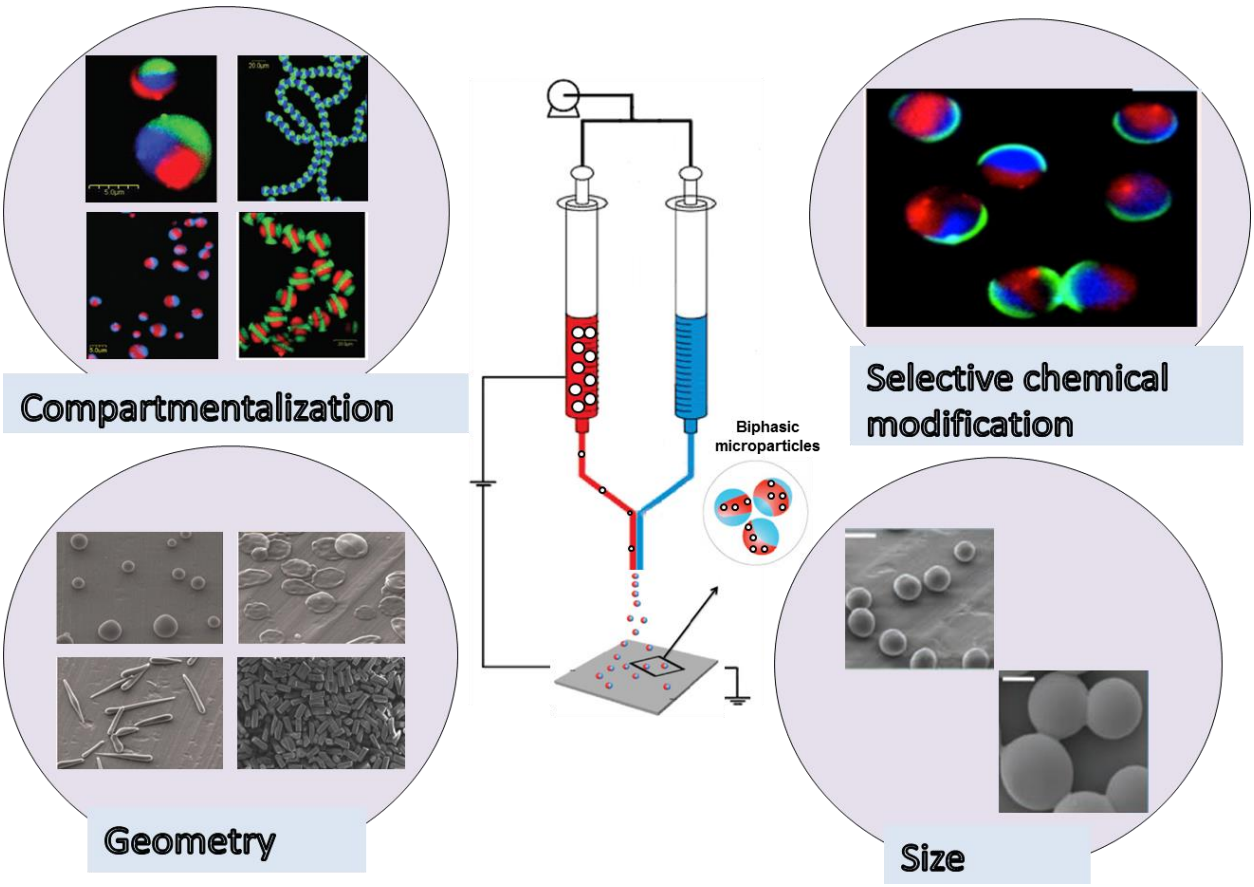


Figure 2-1. Schematic of electrohydrodynamic co-jetting set-up and the particle characteristics that can be controlled through the use of this fabrication process. Adapted from (Bhaskar et al., 2008; Lahann, 2011).

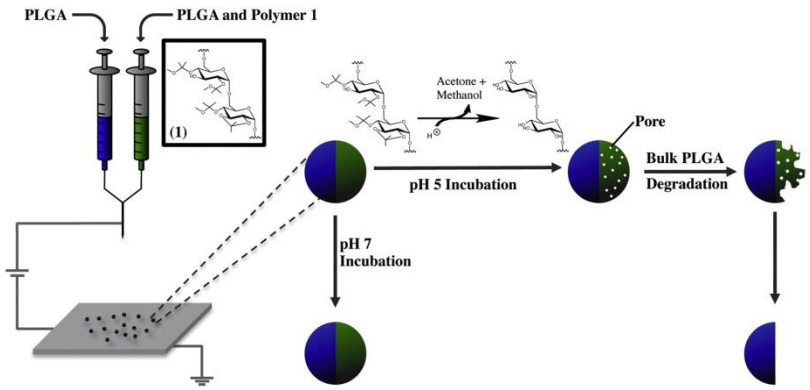


Figure 2-2. Schematic of high density particle fabrication (Rahmani et al., 2013).

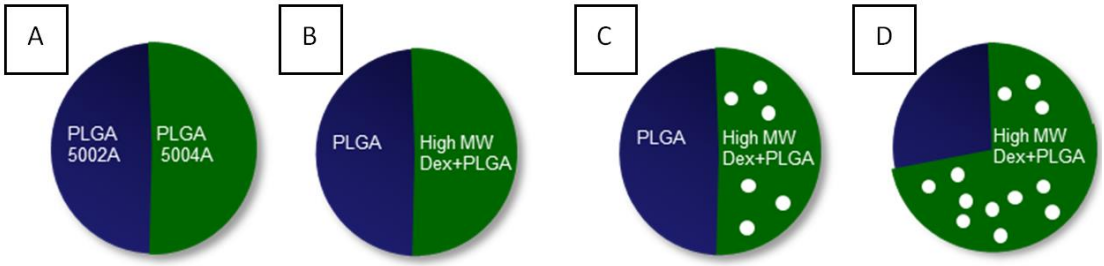


Figure 2-3. Schematic of PLGA hybrid and PLGA/dextran acetal high density particles. White dots indicate pores within the PLGA/dextran compartments. A) PLGA particle comprised of two PLGA polymers of two different molecular weights B) High density control particle (HDOC) C) High density monoporous particle (HDO) D) High density biporous particle (HDOB).

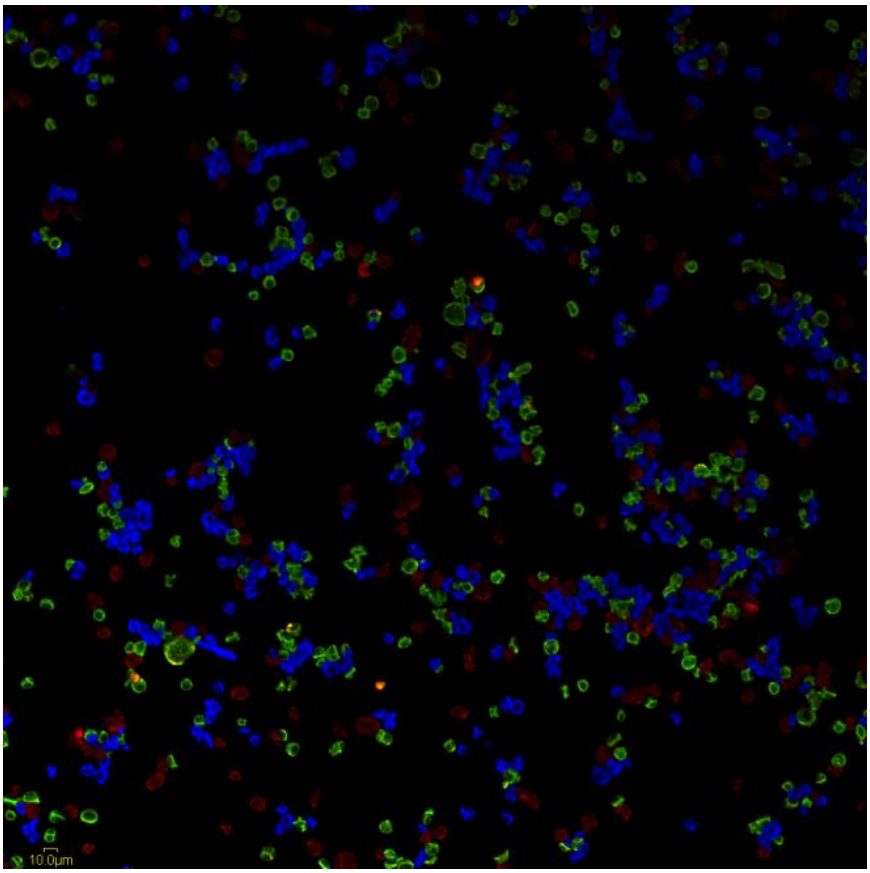


Figure 2-4. 20mg/mL infusion solution of high density microparticles (ratio of particles is 1:1:1 HDO:HDOB:HDOC). The fluorescence is indicative of each particle type: Green=HDO; Red=HDOB; Blue=HDOC.

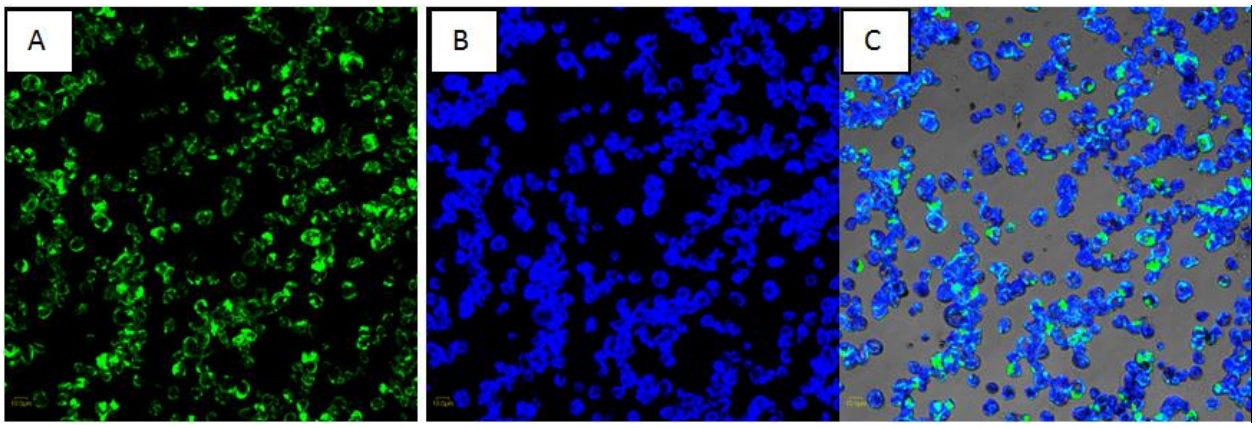


Figure 2-5. High density particles. A) PLGA only compartment B) PLGA/dextran compartment C) PLGA only and PLGA/dextran compartments are distinct.

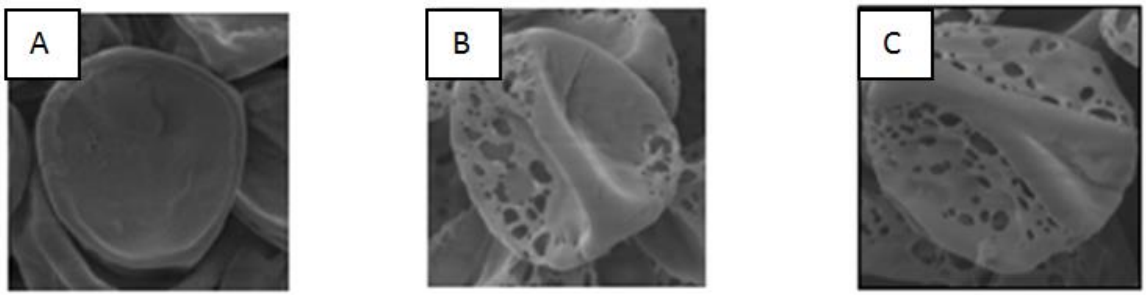


Figure 2-6. Scanning electron microscopy of PLGA/dextran acetal high density microparticles. A) Control B) Monoporous C) Biporous. Images Courtesy of Sahar Rahmani (Rahmani et al., 2013).

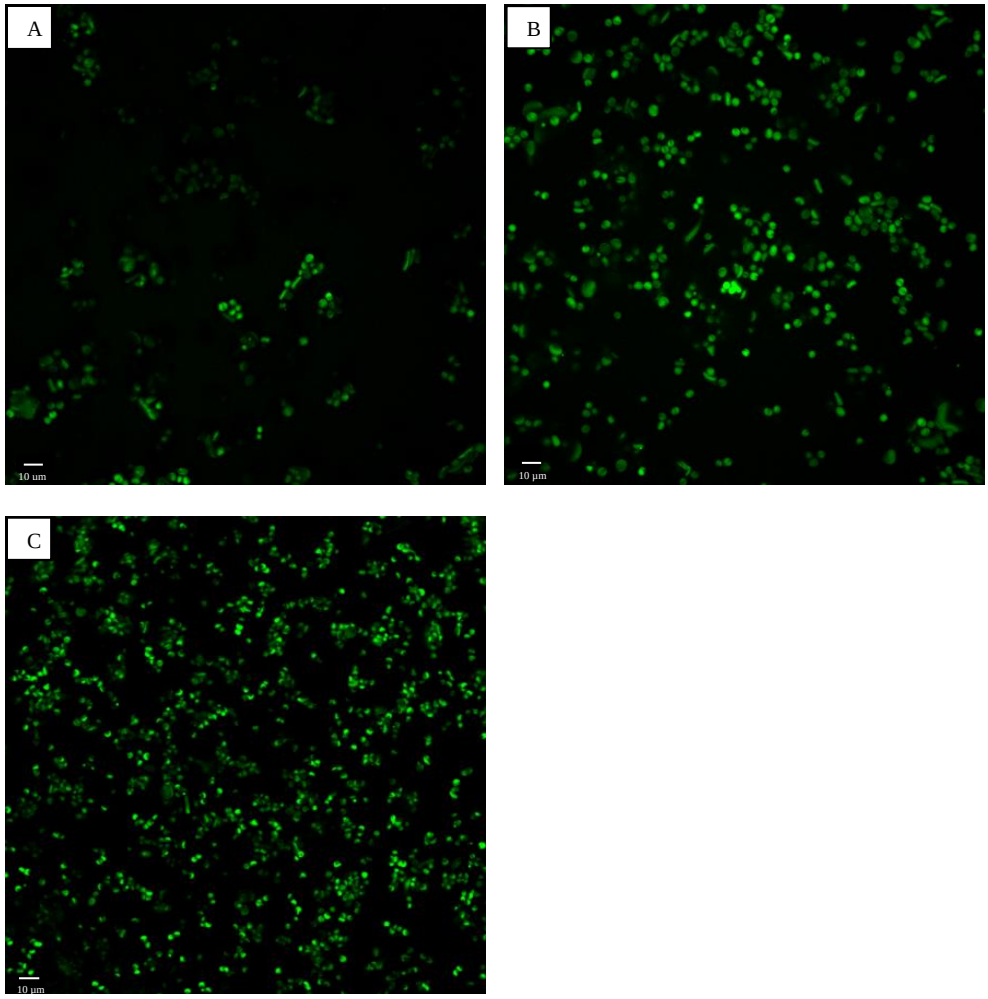


Figure 2-7. Microparticle concentrations (mg/mL) of PLGA hybrid particles. A) 20 mg/mL B) 40 mg/mL C) 100 mg/mL.

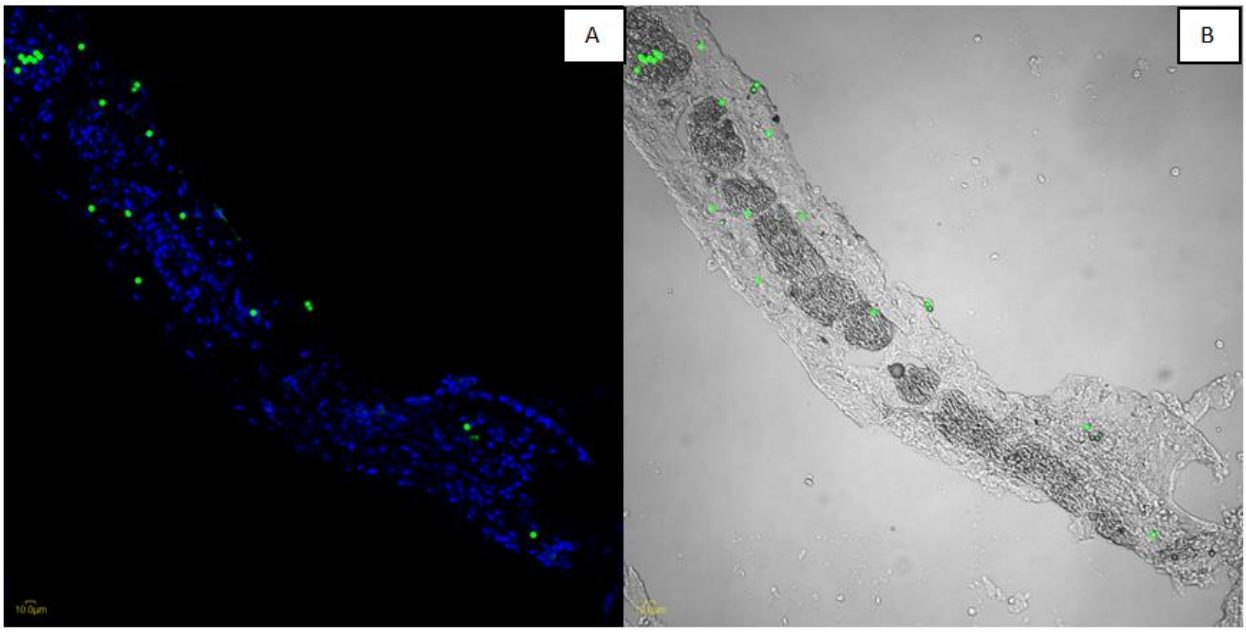


Figure 2-8. Fluoresbrite beads within cochlear tissue. Fluoresbrite beads (green) were easily visible within cochlear cross-sections of the first turn. In this image beads are associated with auditory nerve and the spiral limbus. A) Fluorescent image with DAPI (blue) counterstain for cell nuclei B) Differential interference image.

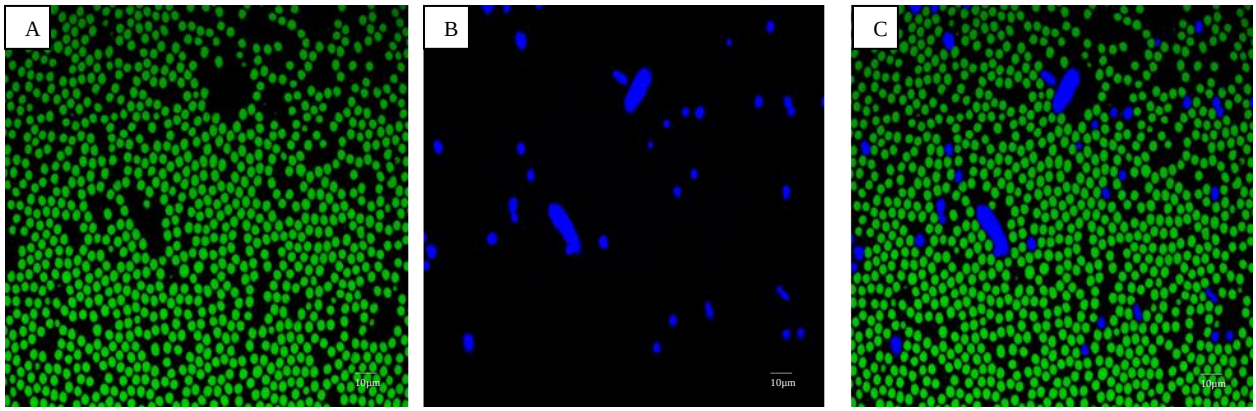


Figure 2-9. *In vitro* comparison of 3 mg/mL blue dye incorporation of MPs with PSBs. A) PSBs B) MPs C) Both .

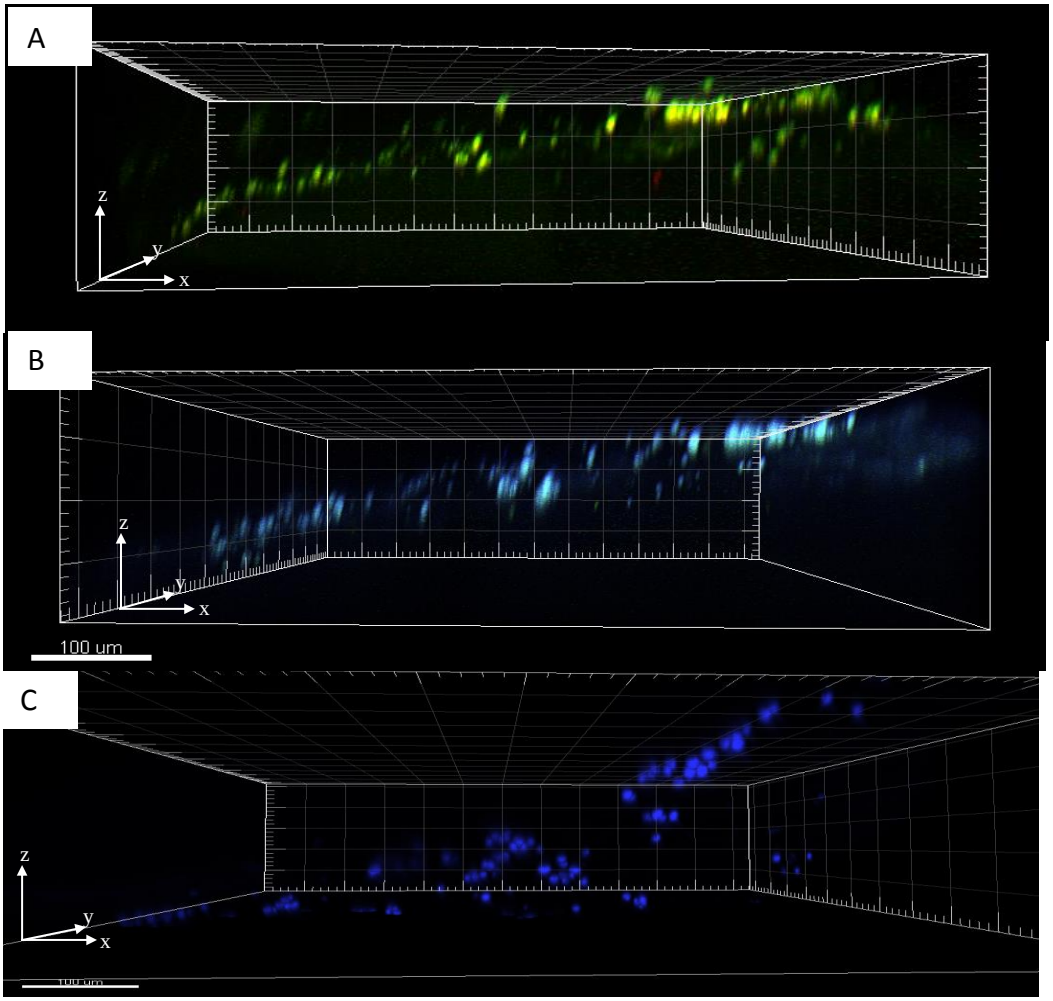


Figure 2-10. 2 Photon confocal laser scanning microscopy image (CLSM) of *in vivo* distribution of particles near round window at 24 hrs in an approximately 250 µm section (z-direction) of intact cochlea. A) PSBs B) Hybrid MPs C) HDOBs.

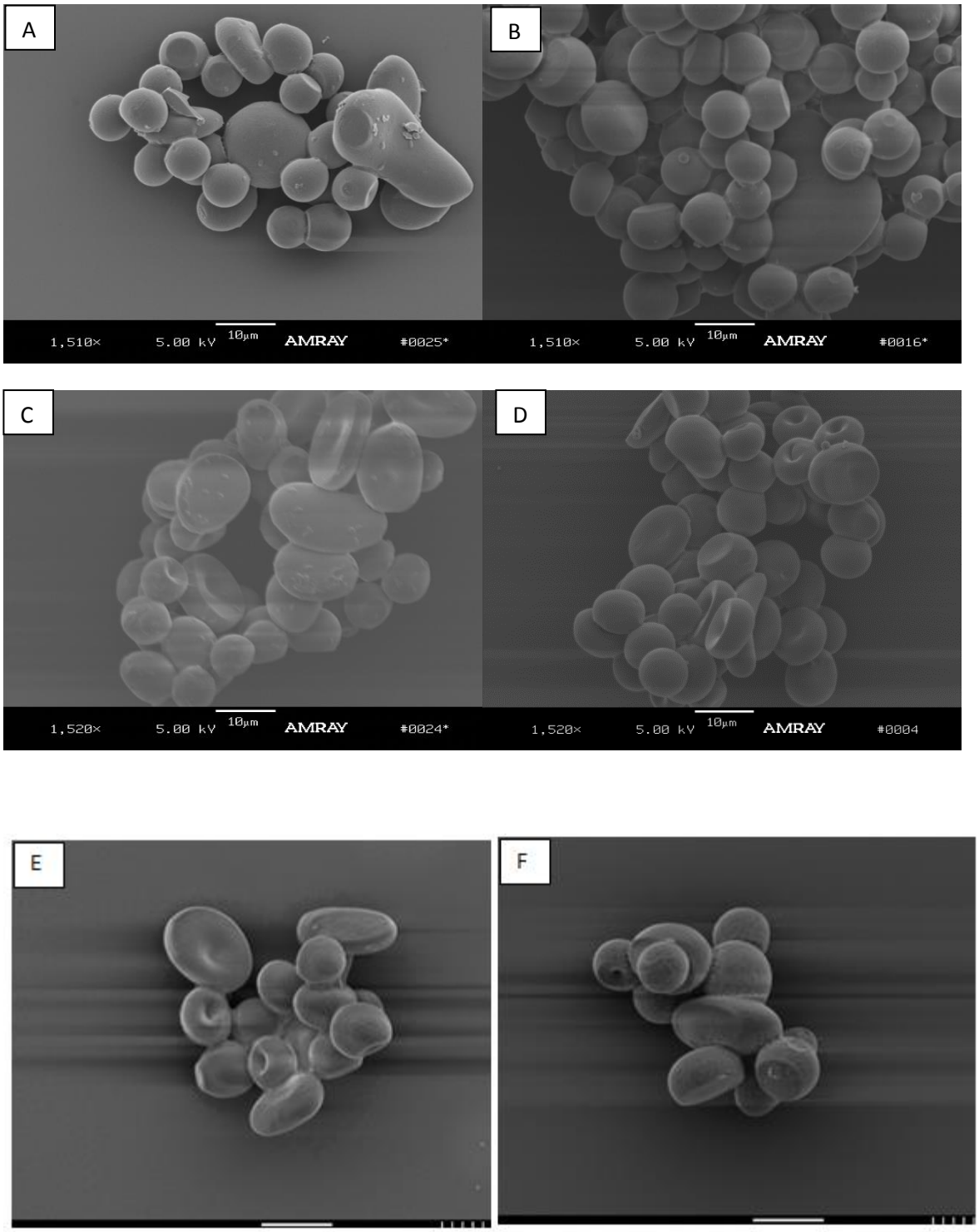


Figure 2-11. Microparticles in experimentally relevant solutions at various timepoints. A) Artificial perilymph at 10days B) EDTA Decalcification solution at 10days C) Artificial perilymph at 6 days D) EDTA Decalcification solution at 6 days E) Immunocal solution at 3hrs F) Immunocal at 4hrs.

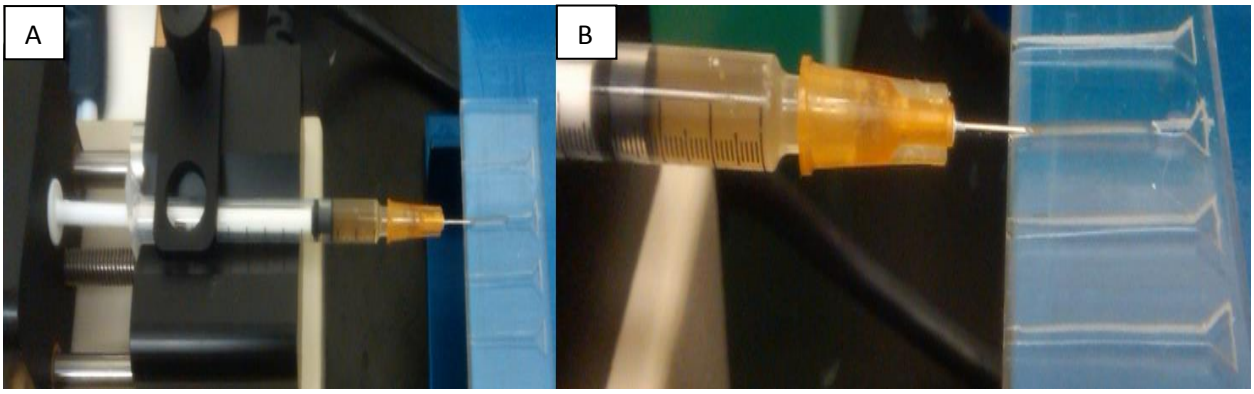


Figure 2-12. Experimental set-up of microparticle distribution within microchannel with cochlear dimensions. A) Syringe pump was used to dispense particles into the channels shown in B). Magnified view of the micropump dispensing microparticle solution into microchannels for assessment.

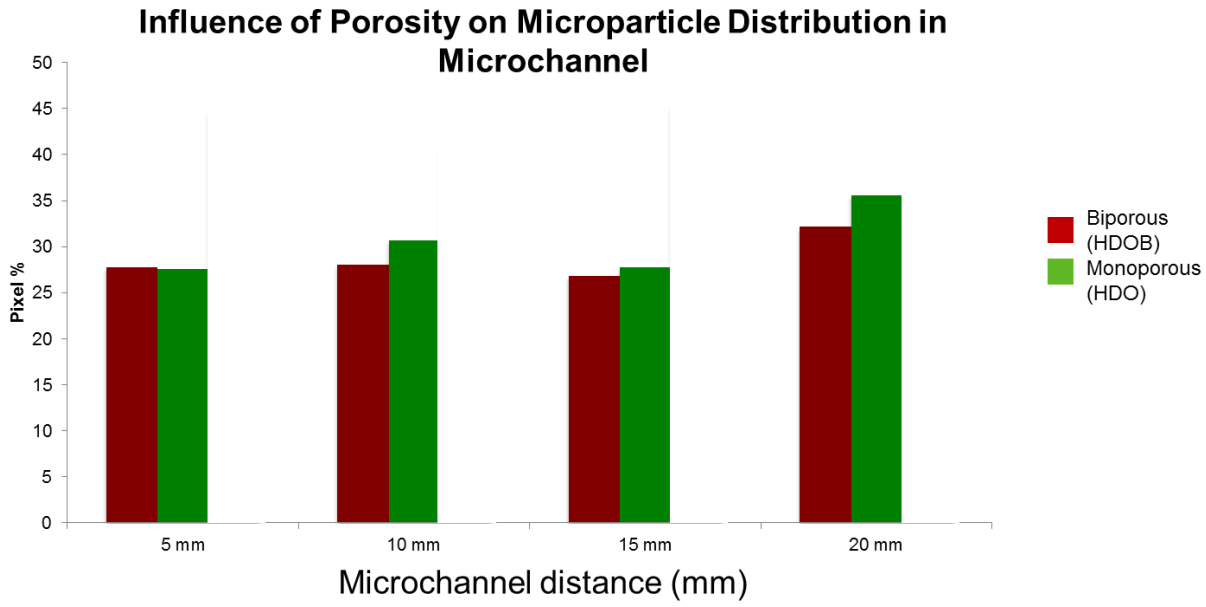


Figure 2-13. Particle distribution within microchannel by particle type. No difference was observed at any of the distances along the microchannel assessed of high density monoporous (green) particles and high density biporous particles (red).

Chapter 3

Particle Distribution, Persistence, and Tissue Tolerance *In Vivo*

Abstract

For patients with moderate to profound sensorineural hearing loss, cochlear implants are the treatment of choice. Increasing numbers of implant recipients have some remaining hearing and this hearing requires protection from the trauma induced by insertion. Local delivery of therapeutic agents could potentially protect remaining sensory cells and neuronal connections. After *in vivo* infusion, cochleae were harvested, decalcified, and sectioned to enable visualization of particles within the scala tympani and cochlear tissue via confocal laser scanning microscopy (CLSM). Microparticles persisted in the cochlea for 7 days post infusion. Quantitative and stereological analysis revealed that at both 1 and 7 days post-infusion, particles were present extending from the cochleostomy site in the base of the cochlea primarily into the first and second turns, and to a more limited extent in the third and fourth turns. The particles' impact on acoustic responsiveness and cell morphology was assessed at day 7 post infusion. Functional assessment was based on auditory brainstem response (ABR) threshold differences between baseline (BL) and day 7 post microparticle infusion . The majority of subjects did not experience a significant BL – Day7 threshold shift, defined as an increase of 15 or more decibels.

Histopathological assessment of microparticle infusion consisted of a cochlear-specific evaluation of hair cell counts (cytocochleograms) and immune response (CD45).

Analysis of hair cell loss included individual graphical representations and statistical analysis. An analysis of variance (ANOVA) was used to facilitate statistical comparisons between the outer hair cell (OHC) loss of treated and untreated ears at each individual turn. OHC loss in turn one, near the infusion site, was not significantly different between treated and untreated ears. In turn 2, the difference was statistically significant; however, average losses for both the treated and untreated ear were well under 10%, the difference may not be as functionally meaningful. Interestingly, turns 3 and 4 were significantly different when treated (left ears) were compared to non-treated (right ears), with higher average losses for treated ears than for untreated ears. CD45, is a marker of white blood cells, and was used to assess white blood cell presence in the cochlea of infused and non-infused ears. No difference was observed in the amount of CD45 reactivity between left ears infused with microparticles and the non-infused right ears that served as contralateral controls.

3.1 INTRODUCTION

3.1.1 Cochlear Implantation and Concerns

Cochlear implants have been a benefit to individuals with hearing impairment for more than 30 years (Waltzman, 2006). The success of cochlear implants as an intervention strategy in individuals with sensorineural hearing loss has led to the expansion of the patient pool for implantation to include those with residual hearing. In this expanded pool, the remaining sensory and neural cells of the cochlea could experience trauma from the physical insult involved in implant insertion. Attempts have been made to modify physical factors involved in implantation such as the use of soft insertion techniques and electrodes designed to minimize structural damage. However, Eshraghi et al. demonstrated that these modifications have not been sufficient to prevent injury on the molecular level (2006; 2007). With the increase of the candidate population for cochlear implantation to include patients with remaining hearing, there is an urgency to eliminate insertion related cell death and thereby hearing losses occurring post implantation (Gantz 2005). Evidence indicates that optimal rehabilitation for these patients would result from use of electrical stimulation from the implant in the dysfunctional areas of their cochleas and amplified acoustic stimulation of the portion with residual hearing and functioning hair cells. This obviously requires the preservation of residual hearing. However, there also is evidence that benefits from electrical stimulation are also enhanced by preservation of residual hearing, including increased speech discrimination, particularly in noisy environments, and sound localization (Mowry et al., 2012).

3.1.2 Approaches to Attenuating Implantation Trauma

Alternative approaches such as the delivery of pharmaceuticals to arrest the mechanisms leading to damage appear necessary to provide protection during implantation. Further, the mode of delivery is crucial to evaluating the efficacy of therapeutic approaches. Because of the blood-labyrinth barrier, local delivery of pharmaceuticals is preferred to enable accumulation of therapeutically effective doses of drug within the cochlea (Alec N. Salt & Plontke, 2009). With preparation of the cochlea for placement of the cochlear implant into the perilymph of the scala tympani, there is an opportunity for local delivery of therapeutic agents that has direct clinical applicability to the primary site of expected trauma. The use of multicompartmental microparticle carriers for drug delivery in the cochlea can provide an excellent way to deliver therapeutics with the potential to protect residual hearing at the time of cochlear implantation. Factors that will determine the utility of employing the carriers for this purpose include the persistence and distribution of the microparticles within the cochlea. Delivery vehicles that can resist clearance from the cochlea and persist on the timescale of weeks to months are needed to provide sustained exposure to therapeutic agents. Chapter 2 contained studies aimed at evaluating the potential to visualize multicompartmental polymeric microparticles within cochlear tissue. This was an important first step to characterizing them as potential drug carriers to facilitate local cochlear drug delivery.

The next steps involve characterizing particle behavior and impact on the cochlea following *in vivo* infusion. This chapter describes studies to evaluate the distribution, persistence, and health impact of microparticles in the *in vivo* cochlea utilizing a guinea

pig animal model.

3.1.3 Rationale for Selected Approach to Attenuating Implantation Trauma

A guinea pig animal model was selected because of the extensive history of its use within auditory research, accumulated expertise in our lab, and surgical anatomy of the head and cochlea which facilitates preparation and microparticle infusion as compared to other rodent models. Multicompartmental microparticles were chosen for this study because of the potential to load more than one drug within a single delivery vehicle. Further by utilizing a system that incorporates biodegradable polymers, the timecourse, and rate of release of each drug from each compartment could be individually modulated based on the indication of the respective encapsulated pharmaceuticals. The biodegradability of microparticles also removes the need to surgically remove the particles at a later date. Microparticle distribution and persistence were assessed utilizing confocal laser microscopy to visualize and image fluorescently labeled microparticles within cochlear cross-sections. This was done at 1 and 7 days post infusion of microparticles into the basal scala tympani. Images were analyzed using quantitative counts and stereological evaluation. Stereological evaluation was incorporated into the analysis of particle presence to extend the evaluation of particle number beyond the particular sections assessed. This method of assessment allowed the projection of the number of particles present within the entire cochlea.

Cochlear functional and histopathological assessments were conducted because it would be desirable for any potential intervention to ameliorate residual hearing loss following implantation to “first do no harm”. The intended application of this intervention was for use during cochlear implantation of an ear with preexisting

pathology. A normal hearing animal model was used to evaluate particle impact on cochlear functionality, cell survival, and cell morphology. The rationale for this decision allowed a more straightforward evaluation and interpretation of particle impact without the confounding factor of deafening. The auditory brainstem response (ABR) was used to assess subjects' responsiveness to pure tones at 4, 8, and 20 kilohertz (kHz). Pre and post infusion threshold sensitivity measures were compared to assess pathophysiology. Functional assessments were performed at 4, 8, and 20 kHz, because it was easily measurable within the research laboratory environment and were located in the more basilar regions of the cochlea near the site of microparticle infusion. Hair cell counts were made to provide quantification and enable graphical visualization of any losses of these important sensory cells. Binning of distances from the cochlear apex into turns facilitated additional analysis wherein hair cell loss per turn could be evaluated to identify whether any differences existed between hair cell loss in infused and non-infused ears. CD45 was used as an indicator of the immunogenicity of the microparticles. Infused and non-infused cochleae were compared for CD45 presence. Although there was a resident white blood cell population, increased numbers of CD45+ cells in infused ears indicated that our microparticle infusion was capable of initiating a foreign body response which could lead to scar tissue formation and even functional impairment, therefore it is best avoided.

3.1.4 Immune Response in the Cochlea

Previously, the cochlea was believed to be an immune-privileged site due to the presence of the blood labyrinth barrier; however, populations of resident macrophages have recently been identified in the stria vascularis and spiral ligament (Eisuke Sato,

2008; Shi, 2010). Though resident macrophages are present within the cochlea, the number of white blood cells present in the normal cochlea is small compared to those present after trauma. This increase occurred within hours of insult, because of an upregulation of intercellular adhesion molecule 1 (I-CAM 1) expression in cochlear cells, which enabled the recruitment of leukocytes from the general circulation (Tornabene et al., 2006). These leukocytes have been identified in the cochlea with markers such as CD45, F4/80, and CD54. The infiltration of these cells leads to inflammation and fibrosis, which can cause degeneration of cochlear cell populations such as those within the spiral ganglion and organ of Corti (García Berrocal & Ramírez-Camacho, 2000). Therefore, any intervention strategy, including the use of microparticles, minimized the immune response of the cochlea during and after application.

3.1.5 Challenges and Considerations for Cochlear Drug Delivery

There are several considerations and challenges concerning cochlear drug delivery. One, the blood-labyrinth barrier, functions similarly to the blood-brain barrier in that it isolates the cochlea from many of the molecules in the general blood circulation. The presence of this barrier makes it difficult for pharmaceuticals delivered systemically to enter the cochlea, let alone to accumulate in high enough concentrations to achieve therapeutic dosing levels (Alec N. Salt & Plontke, 2005). Conversely, the blood-labyrinth barrier aids in preventing systemic side effects when local drug delivery is used to treat cochlear pathologies. Additional challenges to cochlear drug delivery are the persistence and basal-apical distribution of pharmaceuticals and/or drug carriers. Distribution is an issue because the native fluid environment of the cochlea is static, thus the primary mode of dispersion of drugs or carriers within the cochlea is via passive diffusion. Continuous

delivery is needed to promote the development of a concentration gradient that favors distribution of the therapeutic beyond the area immediate to that of the site of initial infusion. Clearance, the removal of pharmacologics and/or carriers from the perilymph of the scala tympani, is another noteworthy challenge for drug delivery to the cochlea. Clearance can occur due to cell binding and uptake, particularly by immune cells, loss to the general circulation via the spiral ligament and stria vascularis, and loss to other cochlear compartments such as the scala media (Salt, 2005). The ideal cochlear drug delivery system would be biodegradable and would deliver multiple drugs with distinct pharmacokinetics, sustain continuous factor release over a defined time period, distribute uniformly throughout the cochlea, and enable targeting of specific cochlear cell populations or functional attachment to the cochlear implant.

Any candidate vehicle for cochlear drug delivery should demonstrate good biocompatibility within the cochlea. Therefore the experiments outlined in this chapter were designed to evaluate the functional and histopathological impact of *in vivo* microparticle infusion on the guinea pig cochlea. Experiments tested the following hypotheses:

Hypothesis 1: Microparticles will be able to persist in the cochlea following *in vivo* infusion. Confocal imaging and quantitative image and stereological assessment were used to analyze the persistence of fluorescently labeled microparticles at days 1 and 7 days following *in vivo* MP infusion.

Hypothesis 2: Microparticle infusion *in vivo* will not inhibit the function and viability of cochlear cells. This hypothesis was investigated at 7 days post infusion by comparing white blood cell infiltration and hair cell survival of infused versus non-

infused cochleae. Day 7 auditory brainstem response (ABR) thresholds were also compared to pre-infusion baseline measurements.

3.2 METHODS

3.2.1 Particle Fabrication

Particles used in the following study were made of poly-lactic- glycolic acid (PLGA) or poly-lactic-glycolic acid and dextran acetal (PLGA/dex). PLGA (MW: 44 kDa) with a lactic to glycolic acid ratio of 1:1 was used and PLGA/dex compartments contained 25% PLGA and 75% Dextran Acetal. All particles were made by electrohydrodynamic co-jetting. Particles containing PLGA/dex compartments were also incubated in an acidic solution (pH=5) for 15 hours to facilitate pore formation on the surfaces of those compartments. Particles were then washed with PBS+1% Tween 20 five times to remove all acid, then filtered through 10 μm filter. After filtration, particles were centrifuged down and the PBS removed. Prior to an experiment, a known mass of particles was suspended in artificial perilymph (AP; 118 mMNaCl, 30 mMKCl, 2.0 mM $MgSO_4$, 1.2 mM $CaCl_2$, 5.0 mM HEPES; pH = 7.35-7.40, osmolality = 285–294mOsm) or artificial perilymph with 55% guinea pig serum albumin (GPSA) to create a 15mg/mL concentration of particles for infusion.

3.2.2 Auditory Brainstem Response (ABR)

Animals were anesthetized with xylazine (10 mg/kg intramuscularly) and ketamine (40 mg/kg intramuscularly). Needle electrodes (active, reference, and ground) were inserted subcutaneously at the vertex and below each pinna and used to record the neurologic response. Up to 1024 responses were averaged for each stimulus level, with the stimulus consisting of a 15-msec tone burst, provided at 10/sec. Pure tones were

delivered via a transducer coupled to the external auditory canal at 4, 8 & 20kHz. Initial sound levels were set at 80 dB for pre- and post- infusion tests. Threshold determination was based on the visual detection of maximum peak–peak amplitude of the resulting waveforms. ABRs were given prior to infusion to enable exclusion of animals with abnormal hearing, and to enable detection, if present, of threshold shifts post infusion.

3.2.3 *In vivo* Infusion

Surgeries were conducted in a sterile environment and utilized aseptic technique. For *in vivo* infusions, Hartley guinea pigs (Charles River Laboratory, Wilmington, MA) were anesthetized, a post auricular approach was used to expose the temporal bone, and provided access to the middle ear. The temporal bone was drilled to visualize the cochlea. A fine pick was then used to create a small, less than 0.2mm, hole in the basal turn of the cochlea 0.5mm from the round window. A micro-cannula with a silastic ball was inserted 0.5mm into the basal turn of scala tympani and cyanoacrylate was used to seal the cannula in place (Prieskorn & Miller, 2000). The microcannula was made from polyethylene 10 (PE10) tubing and polyimide. The silastic ball was made from Sylgard (Dow Corning,Midland, MI). A syringe infusion pump (Harvard Apparatus**,** Holliston, Maine) was used to deliver particles to the scala tympani at a flow rate of 1µl/minute over 5 minutes as seen in **Figure 3-1**. This rate was selected because it is the highest infusion rate that may be used in the cochlea without damaging cochlear cells and structures. Infusions were always performed on the left ear and the right ear was used as needed for a contralateral control.

3.2.4 Cochlear Dissection for Preparation of Cryosections

Guinea pigs were anesthetized and euthanized by injection of sodium pentobarbital (FatalPlus; Vortech Pharmaceuticals, Dearborn, MI). In all cases, secondary euthanasia was performed by transecting the aorta and the ventricle. Animals were decapitated and skulls were opened along the midline to facilitate removal of the brain. Both temporal bones that encase the cochlea were removed. Excess bullar bone was removed to facilitate visualization of the cochlea. The malleus, incus, and stapes bones were also removed. Specimens were fixed in 4% paraformaldehyde for 1-2 hours.

3.2.5 Preparation of Whole Mounts

Guinea pigs were anesthetized and ears were harvested in the same manner as those used for cryosection preparation with a few notable differences. Following removal of the middle ear bones, the apex was visualized under stereoscopic magnification and slightly perforated with a 28G needle to create a small hole. Then the round window was opened and approximately 300µL of 4% PFA was infused directly into the cochlea via the hole in the apex. Specimens were postfixed by immersion in 4% PFA overnight. The following day, cochleae were rinsed and the otic capsule, lateral wall, and tectorial membrane were carefully removed. Phalloidin was used to stain the modiolous as well as the attached organ of Corti. After rinsing to remove excess stain, the organ of Corti was dissected from the modiolous and each turn was mounted and coverslipped onto a microscope slide.

3.2.6 Cytocochleogram

Phalloidin staining of the organ of Corti enabled visualization and counting of both inner and outer hair cells (as indicated by the presence of nuclei and/or stereocillia). The counts were performed on an epifluorescent microscope (Leitz) using the 50X oil

immersion lens and 0.19mm reticules. The most apical turn was counted first followed by the remaining turns in order from those closest to the apex to those farthest from the apex. Counts were recorded as either: all cells present, all cells absent, the number present, the number absent, or uncountable (e.g., folded tissue). Counts were then plotted using Cytogram, a custom software, and presented as percentage hair cell loss at a particular distance from the apex as compared to a database of normal guinea pigs (those not exposed to any external stimuli or agents that could induce hearing loss). The tracking of distance along the cochlear spiral also facilitated the correlation of areas of loss with known frequency maps of the guinea pig cochlea. This provided insight on areas that may be functionally affected by the treatment. While the graphs generated by the aforementioned assessment provided a visual means to qualitatively compare losses between animals and/or treatments, a quantitative means of analysis was required to determine whether significant differences existed. An analysis of variance (ANOVA) was performed using the statistical package for the social sciences (SPSS) software from IBM. A two factor model, with factor 1= ear and factor 2=turn was employed to assess whether any observed differences in loss were significantly different between treated and untreated ears and between turns. Further bonferroni post-hoc correction was used to account for the multiple comparisons made. The p value for significance was .05.

3.2.7 Decalcification and Cryoprotection of Specimens

Following fixation, cochleae were decalcified in a solution that was two-thirds formic acid (Immunocal; Decal Chemical Corporation,Tallman, NY) and one-third 7% sucrose overnight. Prior to freezing, specimens were placed in aluminum containers and immersed in a 30% sucrose solution. Freezing was performed by placing the bottom of

the container in contact with liquid nitrogen cooled 2-methyl-butane (Fisher Scientific, Pittsburgh,PA). Specimens were wrapped in parafilm and stored at -80°C until sectioning.

3.2.8 Cryostat Sectioning

Samples were cut into 14µm sections. For stereological samples, the cochleae were sectioned up to a depth of approximately 4000µm. Every 6th section was collected. A random number generator was used to select a number between 1 and 6. The number generated identified the first slide for analysis in each cochlea. Thereafter, every 6th slide was evaluated such that the slides with numerical markings of 1, 7, 13, 19, etc. were exhaustively assessed. A total of 61 slides were generated for each animal and 10 slides from each animal were assessed to ascertain particle number and distribution. For immunohistochemistry, up to 8 midmodiolar sections were taken from the MP infused cochlea of each animal. These sections were stained with CD45, a leukocyte antigen, to denote immune cell activity, and propidium iodide to indicate the presence of more general cell structures such as nuclei. Cryosections of the liver of one of the guinea pigs were also made for use as positive controls and negative (in the absence of primary antibody) controls.

3.2.9 Preliminary Particle Presence and Distribution Assessment

Preliminary persistence and distribution assessments were conducted using cryosections from cochleae that had particle infusions either 1 or 7 days prior to harvest of the cochlea (n=3 for each timepoint). Sections from various depths of the cochlea were sampled for particle number. The guinea pig cochlea consists of four turns with 2 perilymphatic compartments, the scala tympani and scala vestibule. During assessment,

the location of particles within cochlear cross sections was noted and their distribution within the cochlea determined. A cochlear cross-section and the respective turns of the guinea pig cochlea are shown in **Figure 3-2**.

3.2.10 Stereological Analysis

Particle counts were performed to ascertain the approximate number of particles infused during the *in vivo* assessments. The counts were obtained by loading a microsyringe and dispensing 5µL at the same rate, 1µL/min, used during *in vivo* infusions. To further account for any particle settling effects, the MP solution was allowed to sit in the micropump for 10 minutes, the average time between solution loading and the start of infusion during the animal surgeries, before dispensing the requisite volume. Counts were made by diluting the 5µL of the 15mg/mL particle solution with 100µL of PBS. This diluted solution was vortexed and 5µL were withdrawn and counted using a hemacytometer. Counts were performed in duplicate. Once corrected for the dilution factor, the approximate number of particles in 5µL of a 15mg/mL solution was determined to be 350,000. Stereological analysis was used to determine particle distribution in animals infused with unloaded PLGA/dex particles (n=3) and to estimate the number of particles entering the cochlea at the time of infusion. In particular, the optical dissector method was used to systematically create image slices that contained particles in tissue at multiple planes within the cochlea. This method is an unbiased method whereby an object is counted the first time it appears in an image. A confocal microscope was used to acquire z-stack image series of particles within cochlear cross-sections. The z-step size, and thereby the dissector height, was 3.6µm. Every 6th slide from each sample was evaluated using an unbiased counting grid. The counting grid is

composed of green inclusion lines and red exclusion lines. The grid was superimposed on top of specimen images and a particle was counted if it was inside of one of the squares in the counting grid or in contact with a green inclusion line as seen in **Figure 3-3**. The total number particles within infused cochlear samples could be estimated through the use of Equation 1.

$$Estimated\ number\ of\ particles\ infused =$$

$$average\ particle\ number * probability\ of\ slide\ selection = \bar{n} * \frac{1}{x} \text{ (Eqn. 1)}$$

3.2.11 Immunohistochemistry

Specimens were prepared in the same manner as those for the cytocochleogram, except the duration of post fixation was 2 hours rather than 12. Then the same protocol was followed as outlined in decalcification and cryoprotection. Midmodiolar sections, 12 in total, were selected from 2 of the animals used for the collection of Piribedil exposed perilymph *in vivo*. These sections were assessed with CD45, a leukocyte antigen, to determine the extent to which particle infusion induced a local immune response. Sections from contralateral control ears and the liver from one of the guinea pigs (in the absence of primary antibody incubation) served as negative controls. Cryosections of the liver were used as positive controls in the presence of CD45 primary antibody. The specimens were pre-treated with 0.3% Triton X-100 in PBS for 15 min followed by PBS rinsing (3x5 min). Each section was blocked for 1 hour with 5% goat serum/ and permeabilized for 30min with 0.3% Triton with 5% goat serum and incubated with a primary antibody for CD45 (AbdSerotec MCA1130 (mouse anti-guinea pig), and PBS (1:50). Plates were incubated in the diluted primary solution overnight at 4°C in a sealed

hydrated chamber. The following day, samples were rinsed 2x10 minutes with PBS prior to a 75 minute incubation in the secondary antibody solution containing Alexafluor 488 goat anti-mouse and PBS (1:250). Plates were rinsed 2x10 minutes in PBS. Next samples were incubated in propidium iodide (Sigma) (1:1000 in PBS) for 5 minutes. Propidium iodide staining of nuclei was used to delineate inner ear tissues, providing a visual reference for the location of inflammatory cells within the section. After rinsing in PBS 3x5 min, specimens were mounted, covered with glass coverslips, and stored in the dark at 4°C until viewed.

3.3 RESULTS & DISCUSSION

3.3.1 Particle Persistence and Distribution

The creation of PLGA/Dex particles with distinct polymeric compartments enabled the characterization of the aforementioned particle system for drug delivery, specifically cochlear drug delivery. The immune response in the cochlea was initiated within 24 hours after insult. Previous work has shown that the functional hearing of animals/guinea pigs exposed to transient stimuli such as light noise or surgery can recover after one week (Puel et al., 1998). Therefore, animals were preliminarily assessed at 1 and 7 days post microparticle infusion to observe both particle persistence and distribution. Additionally, the impact of the microparticles on the functionality and histopathology of the cochlea was assessed on day 7.

On day 1 following microparticle infusion, untargeted unloaded particles were distributed in the cochlea as shown in **Figure 3-4**.The majority of the particles were in the first turn of the cochlea (88±17%), followed by the second turn (10±2.0%) and the third and fourth turns (2±0%). The third and fourth turns were combined during analysis

due to the relatively few numbers of particles found in each turn alone. The considerable aggregation of particles in the first turn was not unexpected as the first turn was the site of infusion and there was negligible flow in the cochlea. The dispersion of particles to other turns relies primarily upon diffusion and the small flow induced by the micropump. On day 7 following microparticle infusion, untargeted unloaded particles were distributed in the cochlea as shown in **Figure 3-5**. Similar to day 1 post infusion, the majority of the particles were located in the first turn of the cochlea (94±17%), followed by the second turn (6±2%) and the third and fourth turns (0%). Statistical analysis (student t-test) demonstrated that there was not a significant difference in the persistence and distribution of particles within the cochlea between day 1 and day 7. This indicated that particles which survived acute clearance (washout via eustachian tube, immune response, etc.) within the first 24 hours, were able to remain in the cochlea for an extended time period. This population of particles would be the ones responsible for delivery of therapeutics to the cochlea. To that end, an estimate of the number of particles remaining was necessary.

3.3.2 Estimation of Total Number of Persisting Particles

In order to facilitate an estimation of particle number, it was necessary to use the analysis method, stereology which utilizes random, systematic sampling. The manner in which samples were collected to facilitate stereological analysis was quite stringent and therefore did not enable the retroactive analysis of the samples discussed in section 3.3.1. No statistically significant difference between particle persistence and distribution in day 1 and day 7 specimens was found in the initial groups of animals assessed. Therefore, stereological analysis was performed on a new set of animals (n=3) at 7 days post infusion to estimate the remaining particle number. These animals had a similar

distribution profile to the previous samples as seen in **Figure 3-6** and the average number of particles persisting after 7 days was 20,000±4000. No particles were found in the contralateral ear, indicating that unintended effects on the contralateral ear from diffusion of the delivery vehicle via cerebrospinal fluid need not be a concern with this system. In order to put the estimated number of particles persisting in the cochlea according to stereological analysis in perspective, the total number of particles infused were determined. The starting number of particles in 5μL of a 15mg/mL solution was determined to be 350,000. Therefore, approximately 5.8% of untargeted particles were retained in the cochlea following infusion. Though this percentage was small, the utility of delivery from particles in this scenario was dependent on the dose of drug needed for therapeutic level and the extent to which the drug was able to be incorporated (weight %) into the particles. The incorporation and release of Piribedil from the multicompartmental microparticles will be experimentally outlined and discussed in chapter 4, the next chapter of this dissertation. Further the low percentage observed could be a consequence of the sample processing required to generate the particle cross-sections used in the analysis. During the infiltration of the decalcifying solution and/or the cryoprotection solution, particles located in the fluid spaces could be displaced, meaning that only particles closely associated with the lining of the cochlear chambers would remain for analysis. Therefore, it is possible that non-adherent particles were simply "washed out" of the system before they could be assessed.

3.3.3 Microparticle Impact on Cochlear Function, Cell Survival and Cell Morphology

To determine the impact of these particles on cochlear health, 'health' was defined in terms of both cochlear cell/tissue appearance and the functional performance of the cochlea following microparticle infusion. With respect to appearance, the presence or absence of hair cells and immune cells was evaluated. Hair cells function as mechanotransducers in the cochlea and enable the fluid movement induced by sound waves to be converted into electrical signals that can be interpreted by the brain. These cells are sensitive to damage from both chemical and physical means and their absence impairs the hearing cascade. To determine whether the infusion protocol and/or the composition of the particles and/or infusion solution had a deleterious effect on hair cells, hair cell counts were made and plotted as a cytocochleograms. Cytocochleograms provided a visual representation of areas of missing hair cells and the location of these cells relative to the apex and base of the cochlea. Loss was indicated as a percentage missing in the current animal as compared to a database of normal animals that have not been exposed to conditions that would impact their hearing. Based on previous studies, an area was considered normal if the hair cell loss occurred intermittently and was 20% or less. In addition, in the Hartley strain of guinea pig used in these studies, it is not uncommon to have animals that have large amounts of hearing loss in the apical third of the cochlea. This is sufficiently far from the lower sixth of the cochlea where the particles were infused to enable the determination of the impact of particle delivery on hair cells/hair cell viability. Two of the animals used for cochlear health assessment were found to have the aforementioned apical hearing loss. However, in all but one case,

infused animals had hair cell losses in all rows of less than 20% in the areas adjacent to the site of infusion as seen in **Figure 3-7**. The 2-factor analysis of variance (ANOVA) on OHC loss determined that both ear and turn losses were significant with a p-value below the α=.05 level in treated ears, with those in higher turns demonstrating greater losses. Subsequent analysis was performed in which comparisons were made between the OHC loss of treated and untreated ears at each individual turn as seen in **Figure 3-8**. OHC loss in turn 1, near the infusion site, was not significantly different between treated and untreated ears. In turn 2, the difference was statistically significant at .001, however, as average losses for both the left and right treated ear were well under 10%, the difference may not be as functionally meaningful. Interestingly, turns 3 and 4 were significantly different when treated (left ears) are compared to non-treated (right ears). This seems unusual in that it is known from the distribution studies conducted previously, that the particles were primarily located in the first and second turns. It is possible that the differences seen are related to an increase in intracochlear pressure induced by the surgical method or the viscosity of the background delivery solution formed from artificial perilymph and guinea pig serum albumin. To alleviate potential pressure changes due to displacement of native perilymph during infusion, an outlet hole could be drilled. In this manner, an exit other than the cochlear aqueduct is provided to facilitate removal of excess fluid from the perilymphatic space.

Auditory brainstem responses (ABRs) were used to assess the hearing of animals pre- and post-infusion. Pre-ABRs were given to select only animals with behaviorally normal hearing (particularly in the lower two-thirds of the cochlea; the upper third was beyond the frequency threshold that can be measured non-invasively) to undergo microparticle

infusion. The assessment of baseline hearing also provided a basis for comparison to ABR results following infusion to assess particle impact on hearing. Post-infusion testing was conducted at the same three frequencies, 4, 8, and 20kHz as the pre-test. If the intensity level required for the subject to detect the stimulatory tone at any of the frequencies changed, then a threshold shift occurred. Hearing was considered to have worsened if the sound intensity needed for the subject to detect the stimulatory tone was 15 or more decibels higher than in the pre-test. This value was selected because it has been shown to be outside the bounds of normal experimental variation (depth of transducer insertion, etc.) and, therefore indicative of an actual functional change in hearing capacity. As seen in **Figure 3-9**, following infusion, three out of five animals had hearing within the normal range as compared to their pre-infusion values which is indicated by the threshold shift. One of the animals, NAN 20, demonstrated a threshold shift (15dB) at one frequency, 20kHz. Another animal, NAN 22, demonstrated threshold shifts at both 4 and 8kHz. The gross anatomy of NAN 22 correlates well with observed functional losses as fluid was present in the middle ear of this animal and excess fluid makes it more difficult for sound to be transmitted, particularly at the lower thresholds. Since the cytocochleogram of NAN 22 demonstrated minimal hair cell loss, functional deficits at the lower frequencies were likely the result of conductive hearing losses due to the presence of the middle ear fluid. Therefore, microparticle infusion was well tolerated in four out of five animals with only one animal NAN 20 displaying functional and histological losses. Further, the number of white blood cells present in treated ears was comparable to that seen in the untreated ears indicating that at 7 days post infusion, the immune response to the particles was negligible as seen in **Figure 3-10**.

3.4 CONCLUSIONS

This work has demonstrated the feasibility of delivering multicompartmental microparticles to the cochlea. Specifically it has characterized the *in vivo* persistence, distribution, and drug release from the chosen particle system. Particles were maintained in the cochlea for at least seven days. The impact of particles on cochlear functionality was minimal and no increase in infiltration of immune cells cochlear cell survival was observed. Further, cell survival and morphology were well maintained in the areas of the cochlea with high numbers of particles. An increase in the number of animals for study of the *in vivo* functional and histological assessments is necessary to provide definitive support for the biocompatibility of multicompartmental microparticles within the cochlea.

3.5 FIGURES & TABLES

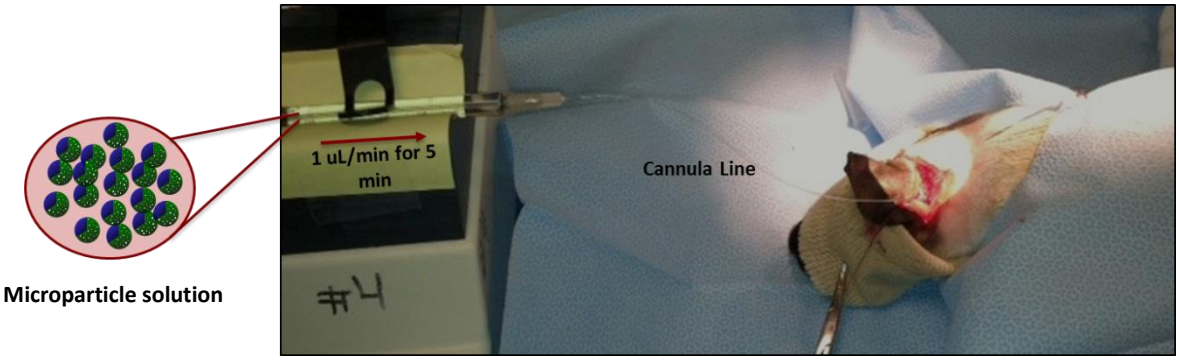


Figure 3-1. Photographic image of the experimental setup for the MP infusion. Intrascalar delivery of microparticles at a rate of 1µL/min is facilitated through the use of a syringe pump, microsyringe, and cannula.

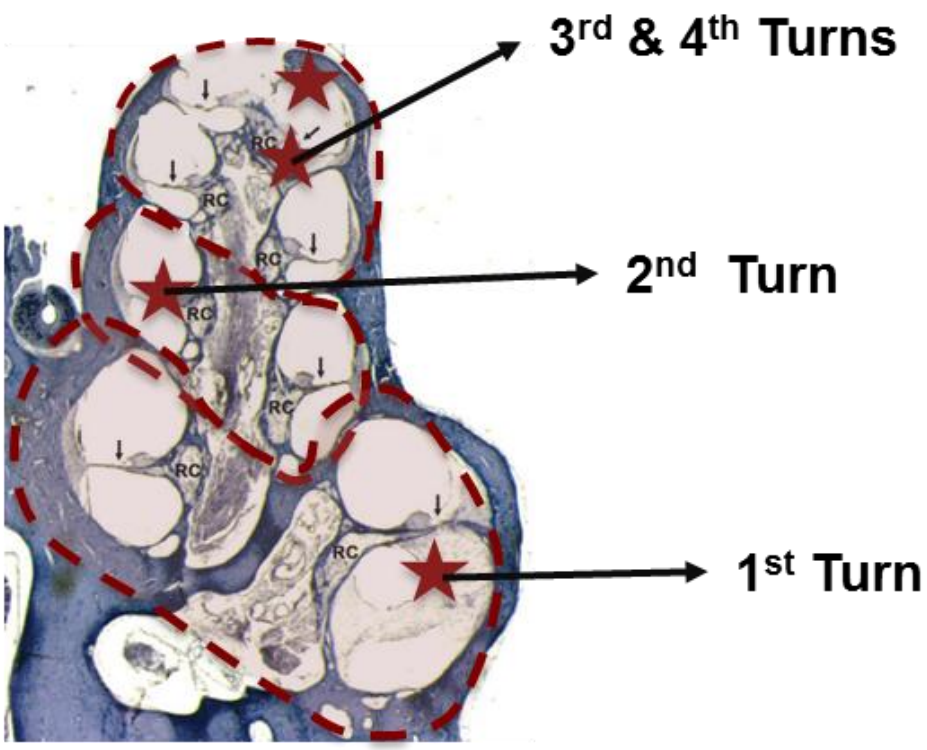


Figure 3-2. Example of cochlear cross section with delineation of the respective cochlear turns in which particles were distributed.

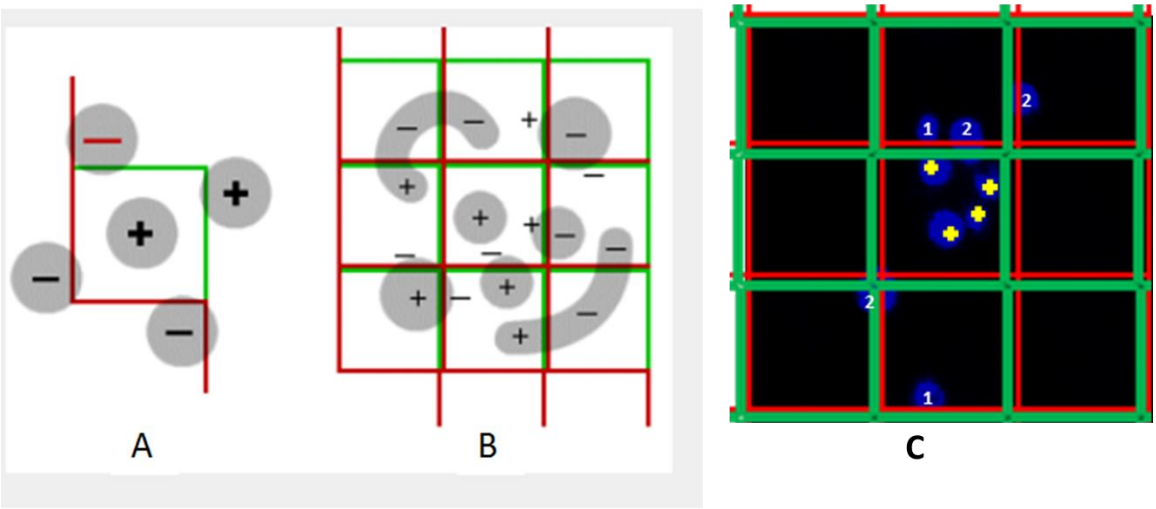
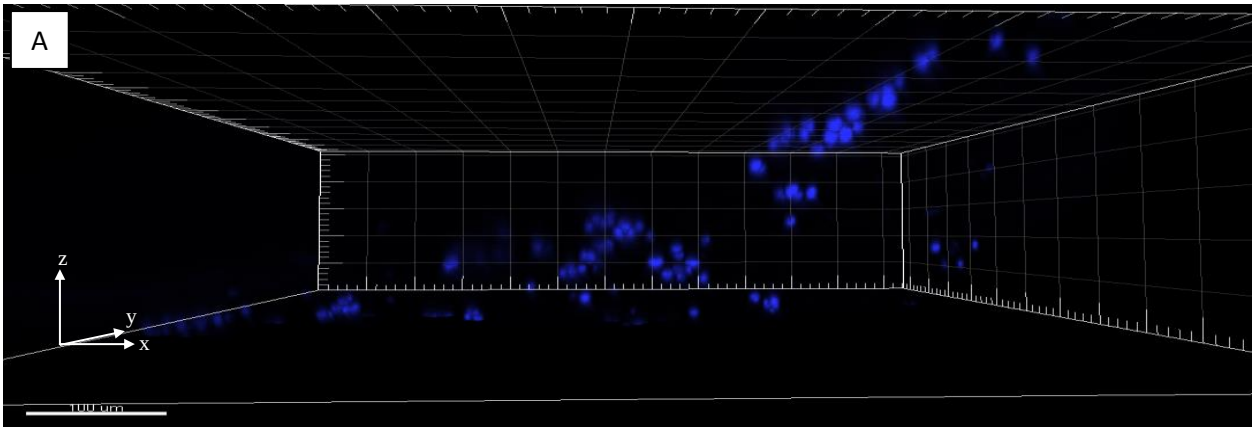


Figure 3-3. Example illustration of inclusion/exclusion grid overlayed on images to facilitate stereological analysis. Pluses indicate objects that would be counted as present while objects with minuses would not be counted. A) Magnified view of a small cluster of objects associated a single cell of the inclusion/exclusion grid. B) View of a cluster of objects within the larger framework of the inclusion/exclusion grid (Slomianka) C) Application of grid to microparticle infused cochlear cross-section. Particles with yellow crosses would be counted while particles touching the red exclusion line (1) and particles outside the square grids (2) would not be counted.



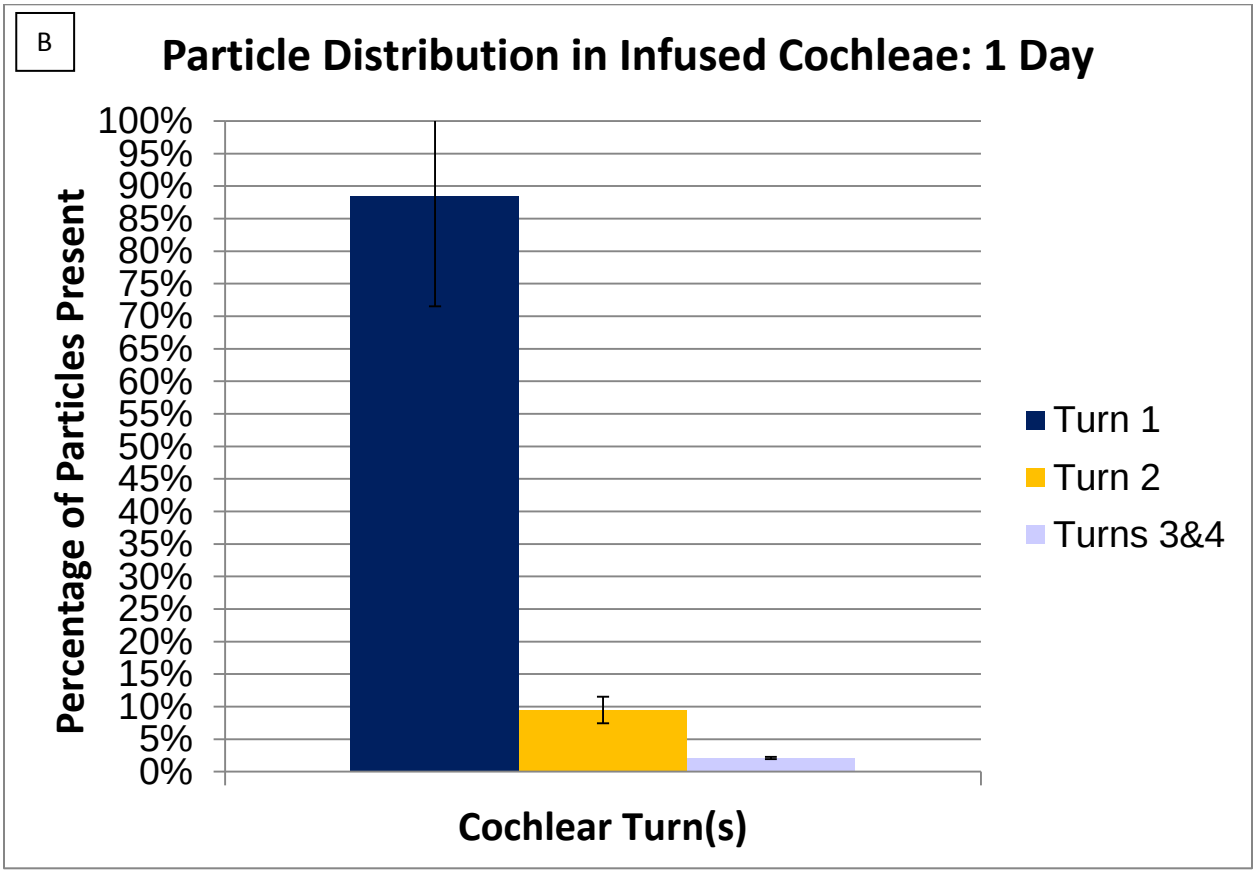
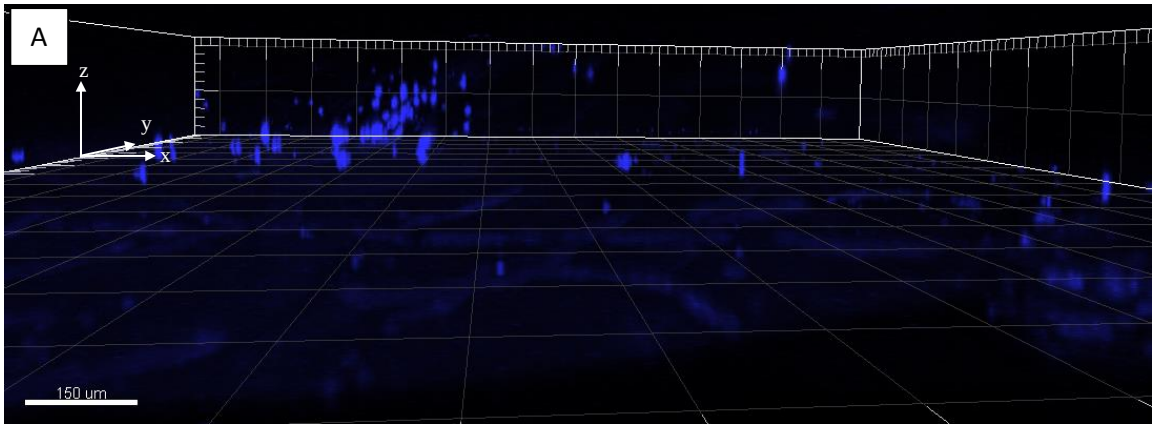


Figure 3-4. Distribution of unloaded microparticles observed 1 day after *in vivo* infusion. (A) 3 dimensional projection of the particles near the round window of the cochlea wherein the basal to apical direction is represented as positive in the x-z plane (B) Distribution of particles within the four turns of the guinea pig cochlea where turn 1 is the closest to the cochleostomy/infusion site.



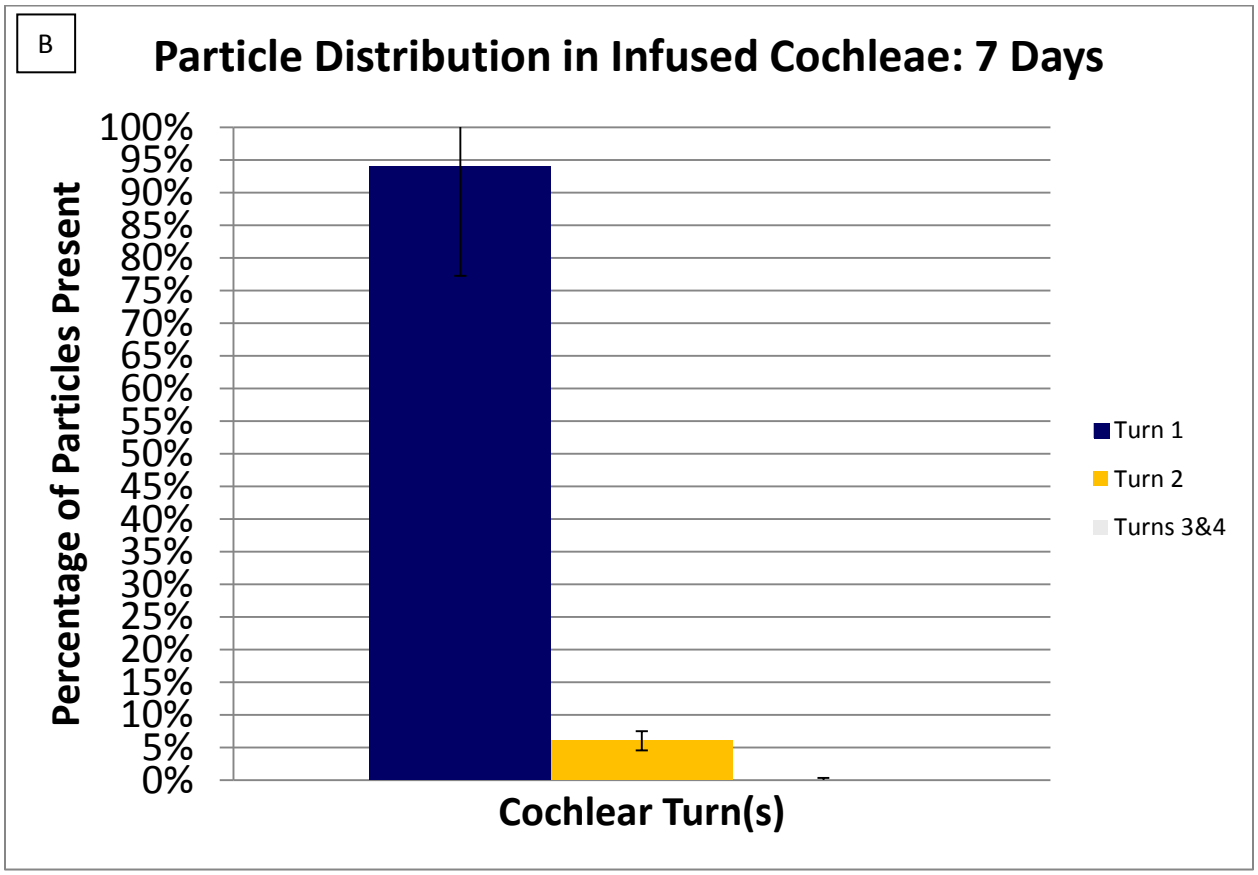


Figure 3-5. Distribution of unloaded microparticles 7 days after *in vivo* infusion (A) 3 dimensional projection of the particles near the round window of the cochlea wherein the basal to apical direction is represented as positive in the x-z plane (B) Distribution of particles within the four turns of the guinea pig cochlea where turn 1 is the closest to the cochleostomy/infusion site.

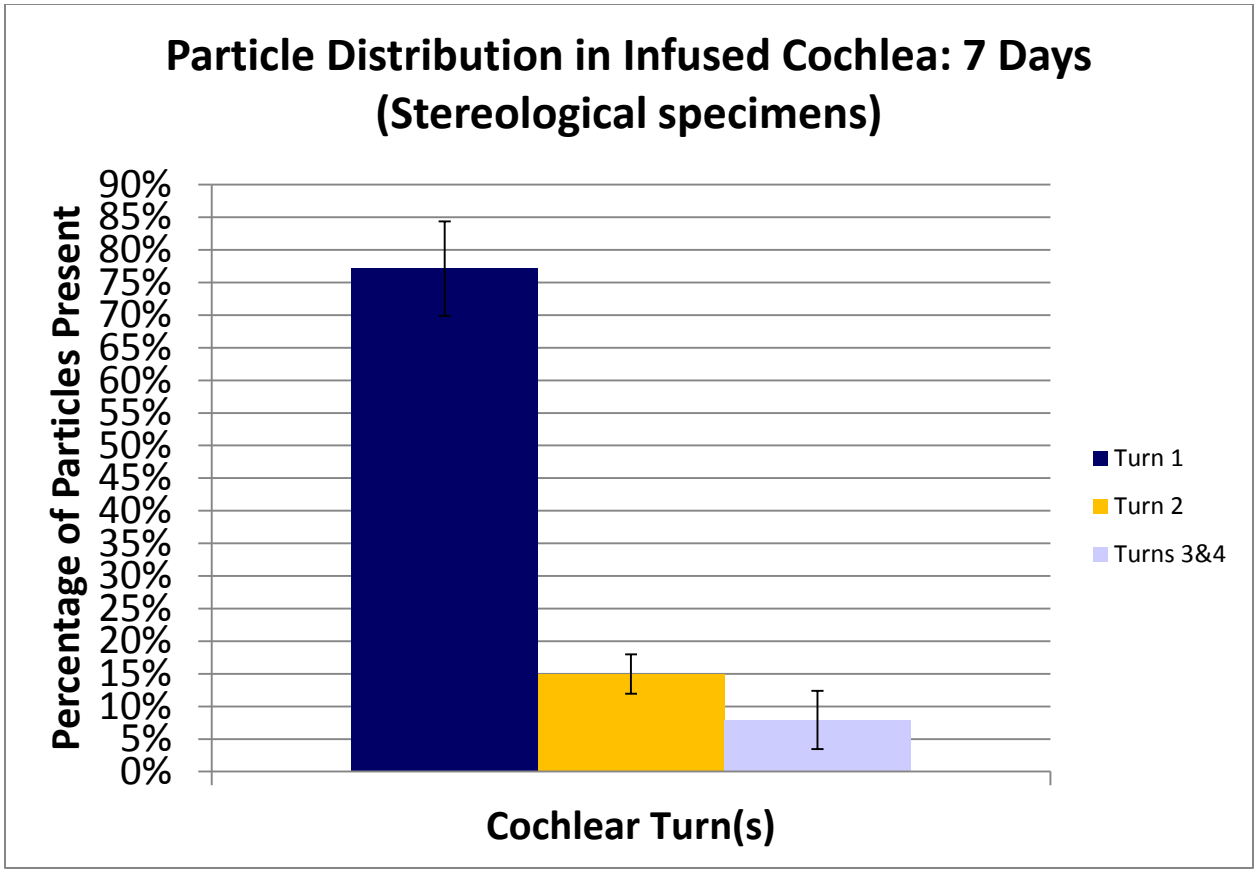
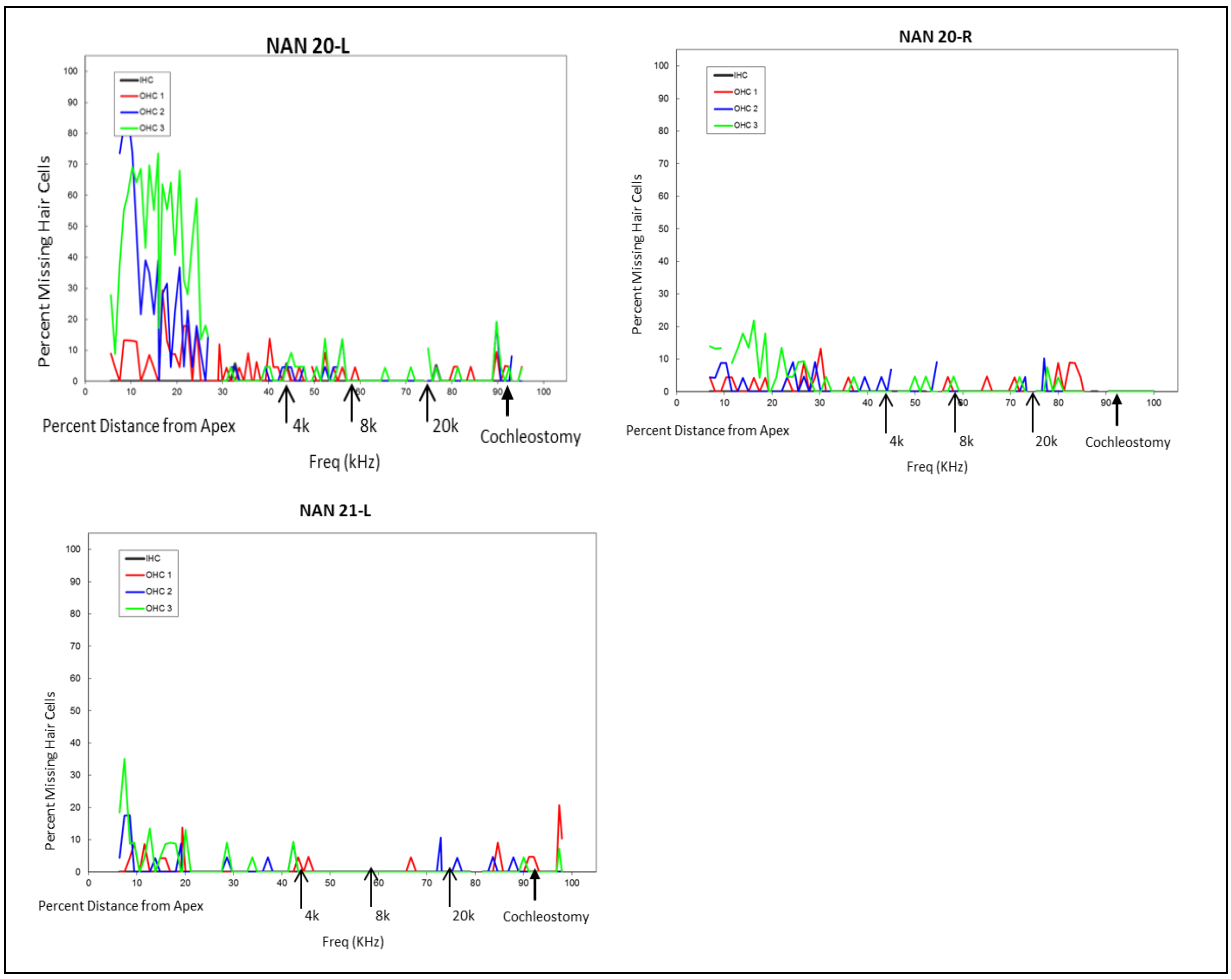


Figure 3-6. Particle distribution of unloaded microparticles observed 7 days after *in vivo* infusion. Distribution of particles within the four turns of the guinea pig cochlea where turn 1 is the closest to the cochleostomy/infusion site.


NAN 20-L
IHC
OHC 1
OHC 2
OHC 3
Percent Missing Hair Cells
Percent Distance from Apex
4k
8k
20k
Cochleostomy
Freq (kHz)
NAN 20-R
Percent Missing Hair Cells
Percent Distance from Apex
4k
8k
20k
Cochleostomy
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NAN 21-L
Percent Missing Hair Cells
Percent Distance from Apex
4k
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20k
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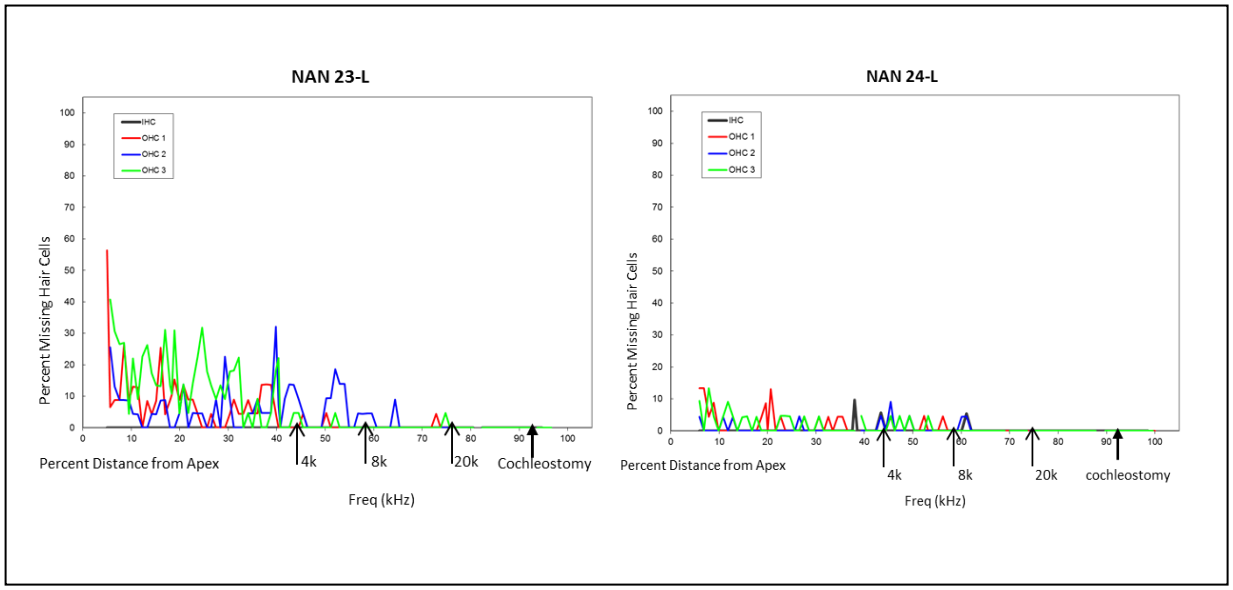


Figure 3-7. Cochlear hair cell death as represented by cytocochleograms (graphical representation of hair cell loss) at day 7 post infusion for guinea pigs (n=5) receiving 5µL of microparticles. The regions of ABR functional testing as well as the cochleostomy site have been indicated along the x-axis. In all animals hair cell loss near the cochleostomy site is minimal.

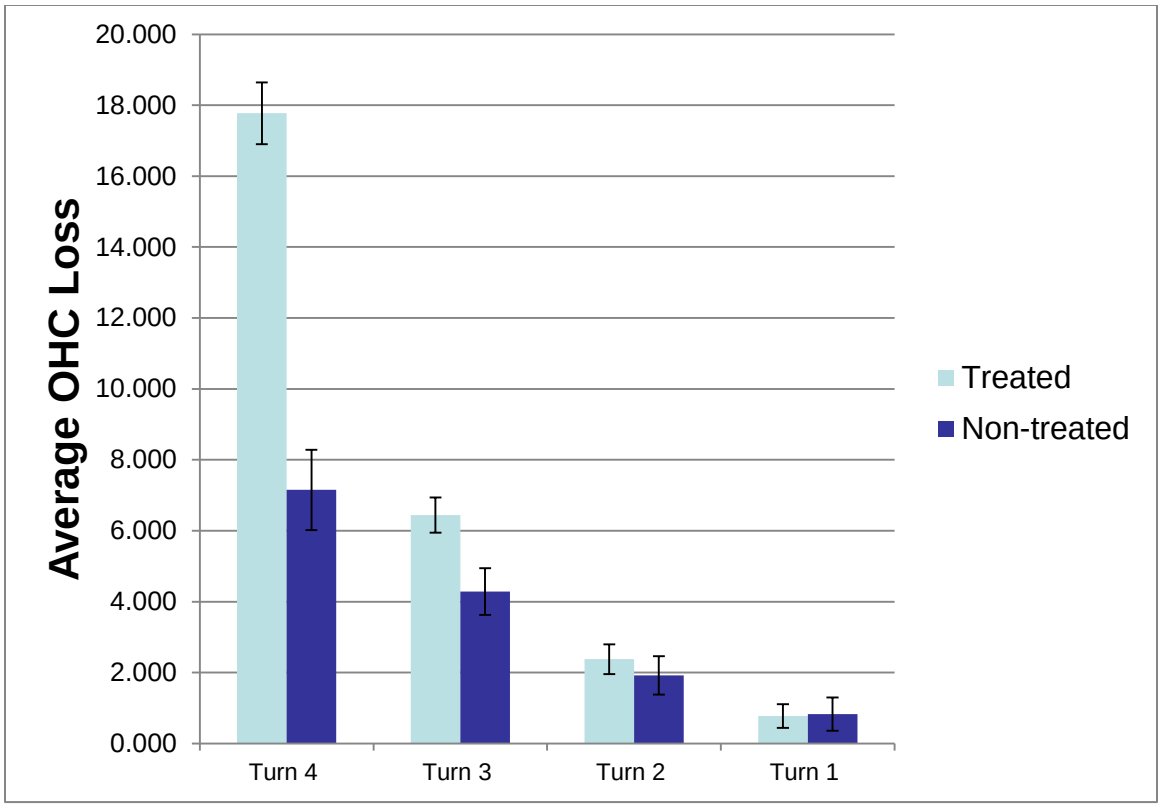


Figure 3-8. A graphical depiction of the OHC loss as a function of ear (treated/left vs. non-treated/right) and turn at 7 days post MP infusion. ANOVA analysis with α=.05 found that turn (p=0.00) and ear (p=0.00) were both significant. The differences between ears were primarily driven by OHC losses in turns 3 and 4.

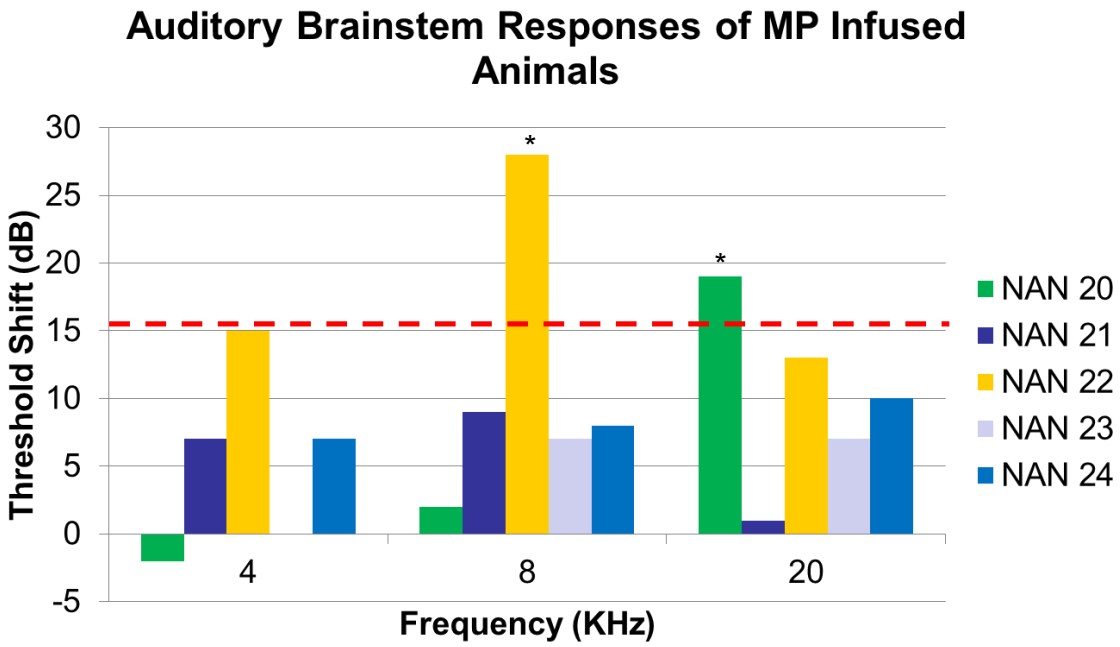


Figure 3-9. Cochlear function 7 days post MP infusion as represented by auditory brainstem responses. For all animals, threshold shift (difference between pre and post infusion thresholds) at 4, 8, and 20 kilohertz are shown. A threshold shift greater than 15 dB is indicative of a change in an animal's hearing. At the majority of frequencies across animals, no shift is observed, though NAN 20 and 22 each demonstrate a shift at 20 and 8 kHz, respectively.

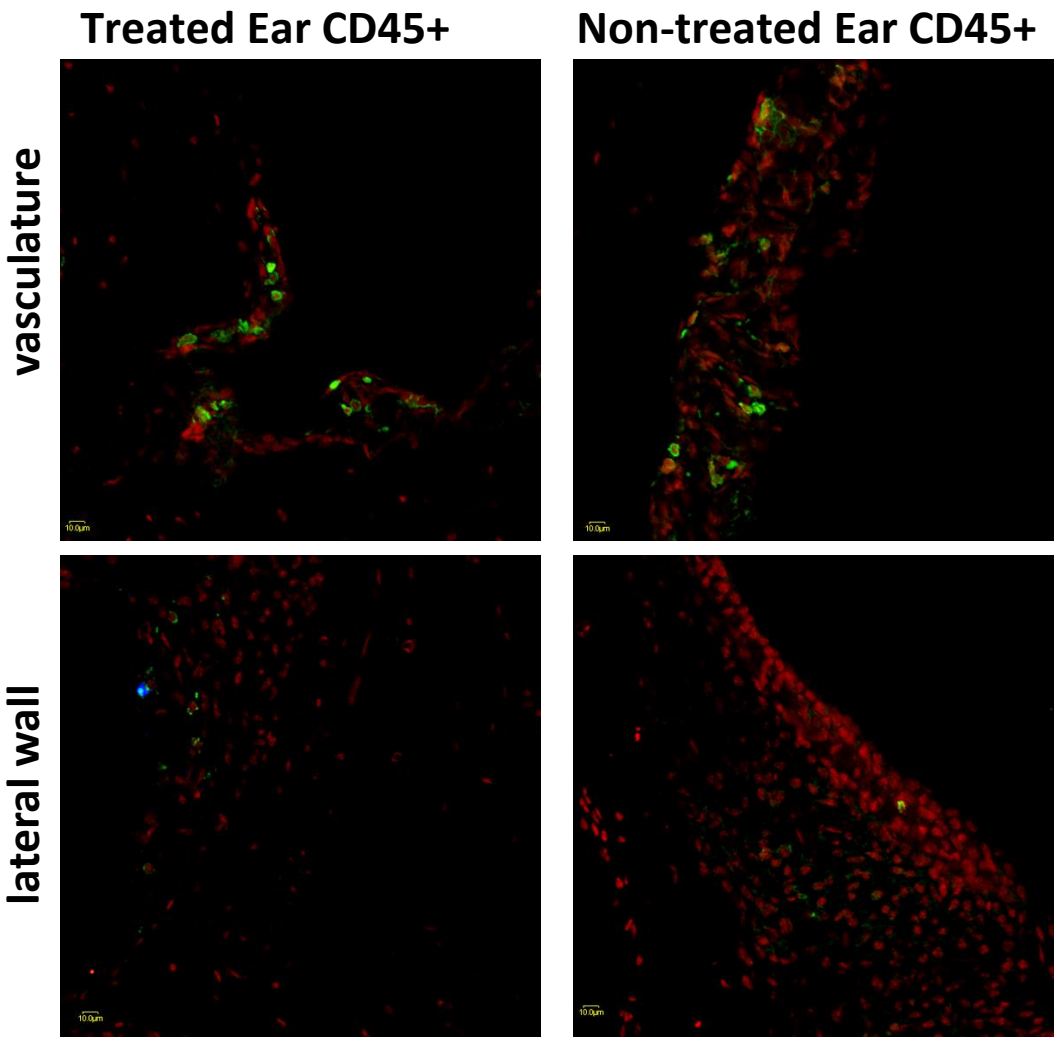


Figure 3-10. CD45 presence in treated and untreated cochleae. Resident white blood cells may be in the normal cochlea and no differences are seen in the number of these cells present at 7 days following MP infusion. Leukocytes cells are identified as nuclei (red) whose surface/cell membrane is positive for CD45 (green).

Chapter 4

Microparticle Release of Anti-Excitotoxic Agent *In Vitro* and *In Vivo*

Abstract

Drug delivery to the cochlea is challenging because of issues related to poor drug accumulation, particularly from systemic delivery. Drug accumulation is needed to enable pharmaceutical concentrations within the cochlea to reach the levels required to provide therapeutic efficacy. However, cochlear implantation and the additional pathology experienced post-implantation by patients with residual hearing provide an excellent opportunity to utilize local drug delivery technology to address a significant clinical problem. This approach could increase patient benefit from implantation while minimizing the effects of pharmaceutical agents on other parts of the body.

To investigate the utility of using multicompartmental particles for intracochlear drug delivery, porous PLGA/dextran acetal microparticles were fabricated for *in vitro* and *in vivo* studies of drug release from microparticles. During jetting, Piribedil (a pharmaceutical demonstrated to reduce pathology from excessive neuronal stimulation in the cochlea) was solubilized in the PLGA contained in the PLGA-only compartment of the microparticle. Confocal microscopy imaging revealed both the incorporation and the sequestration of the Piribedil within the PLGA-only compartment of the PLGA/dextran acetal microparticles. Ultraviolet (UV) spectrophotometry was then used to characterize the *in vitro* release profile of the Piribedil from monoporous and biporous microparticles over a 14-day period. Both particle types exhibited sustained release and no differences

were observed in the release profile of monoporous particles as compared to biporous particles. This latter finding provides independent secondary confirmation of the successful incorporation of the Piribedil solely within the non-porous PLGA-only compartment. *In vivo* assessment of perilymph (inner ear fluid) by liquid chromatography mass spectrometry (LC-MS) revealed that Piribedil release from biporous particles was detectable at 7 days post infusion. Further, *in vivo* Piribedil concentration reached therapeutic levels in some animals.

4.1 INTRODUCTION

The previous chapters evaluated microparticle visualization, persistence, distribution, and impact on cochlear health in *ex-vivo*, *in vitro* and *in vivo* systems. In this chapter the encapsulation and release of a relevant therapeutic, Piribedil, from the multicompartmental microparticles is assessed both *in vitro* and *in vivo*. Piribedil was selected as a pharmaceutical of interest because one possible mechanism involved in neural cell death following cochlear implantation is excitotoxicity. This excitotoxicity results either from inner hair cell apoptosis leading to deterioration of the inner hair cell-auditory nerve (IHC-AN) connection or excessive glutamate release from the IHC onto the IHC-AN synapse causing bursting of auditory nerve processes (A. A. Eshraghi, and T. R. van de Water, 2006). Piribedil is an FDA approved pharmaceutical that has previously been shown to act as an anti-excitotoxic agent in the cochlea (d'Aldin, 1995). The classification of Piribedil as an FDA approved drug, would likely hasten the time to clinical application and was a consideration in its selection for this study. In addition, Piribedil has an innate fluorescence in the 200 to 300 nanometer wavelength range which would allow detection of the drug within the polymer via confocal microscopy in *in vivo* studies and UV spectrophotometry in *in vitro* release studies. An additional approach was necessary to enable the assessment of *in vivo* particle release since native perilymph is a volume limited and potentially biologically complex fluid. Therefore, liquid chromatography mass spectrometry (LC-MS) was chosen because of the enhanced sensitivity and specificity needed to detect Piribedil in the microliter quantities of native perilymph obtained from *in vivo* sampling. The sensitivity of this assay is increased by the incorporation of mass spectrometry which is capable of measuring analytes even at very low concentrations (nanomolar and even picomolar range). Since the concentration

of drug present in the scala tympani was unknown and difficult to predict due to potential loss via clearance to the bloodstream or other cochlear compartments, we selected a method of analysis that would enable detection of Piribedil even at very low concentration. In addition, increased specificity was provided by the use of mass spectrometry in that compounds are not only distinguished by their retention time, but also by their mass and the pattern in which they fragment inside the mass spectrometer.

Encapsulation and sequestration of Piribedil within the multicompartmental microparticle was first performed. Once done, *in vitro* assessments of release were performed to create a release profile of Piribedil from the microparticles at various timepoints. This profile defined the extent to which Piribedil release from the microparticles could occur in the controlled, sustained manner required for intracochlear drug delivery. Further *in vivo* testing of Piribedil containing microparticles was performed to assess whether particles could release detectable and therapeutic amounts of Piribedil in perilymph and surrounding target cochlear tissues.

4.1.1 Anti-Excitotoxicity in the Cochlea

Anti-oxidants, corticoids/anti-inflammatory agents, and anti-excitotoxic agents have all been explored as potential pharmaceutical interventions for inner ear pathologies. Of the three classes, anti-oxidants have been the most widely investigated because of their demonstrated ability to preserve hair cells from trauma. Because of the focus on hair cells, anti-excitotoxic agents have not been as extensively explored. The use of this class of therapeutic could complement hair cell sparing strategies by sparing neurons which are also critical components of the inner ear hearing cascade. Therefore, an anti-excitotoxic agent was selected for further study. The anti-excitotoxic pharmaceutical chosen to

establish this treatment paradigm was Piribedil, a dopamine receptor agonist. In the presence of increased neural activity, NMDA receptors, which are blocked by magnesium under normal conditions, become responsive to the release of the neurotransmitter glutamate. As a result of this increased responsiveness, an excitotoxic environment can be induced wherein auditory neurons are overstimulated causing their ion channels to open excessively as seen in **Figure 4-1**. Excessive opening of the ion channel can lead to increased calcium influx and ultimately neuronal calcium overload and cell death (Mark et al., 2001).

A strategic approach in the field has been to attenuate excitotoxicity by preserving inner hair cells (IHCs) and subsequently the inner hair cell auditory nerve (IHC-AN) connections and neurons (Le Prell, 2007). Successful reconnection of neuronal processes with IHCs following insult was demonstrated in noise studies by (Puel et al., 1998), as long as the IHCs were maintained, leading to the assumption that the presence of IHCs indicated the presence of functional IHC-AN connections, as seen in

Figure 5-1. **Outline** of **experiment to determine** efficacy of local Piribedil delivery in response to trauma such as noise. The duration of each phase of the experiment is indicated as well as the timing of functional assessments. However, more recent studies by (Kujawa, 2009) demonstrate that significant losses of IHC-AN connections can be observed even when IHCs are preserved, indicating that the reconnection previously observed may not be as efficient as initially thought.

Prior studies by (d'Aldin, 1995) using 6-hydroxydopamine and ischemia to challenge the cochlea demonstrated the postsynaptic regulation of primary auditory neurons by

lateral efferents from the auditory brainstem. The authors found that this regulation was made possible by axodendritic synapses between the lateral efferents and the afferent peripheral processes of the auditory neurons in contact with the inner hair cells. Specifically, this study demonstrated that dopamine may be involved in inhibitory regulation at the synaptic connection between inner hair cells and auditory neural processes. As a dopamine receptor agonist, Piribedil should inhibit the synapse connection between inner hair cells and auditory neurons, thereby reducing excitation without inducing serious side effects such as hearing loss. In the study by (d'Aldin, 1995), the authors found that intracochlear delivery of Piribedil prevented the swelling and bursting of peripheral processes otherwise induced by transient ischemia. In another acute by d'Aldin study evaluating Piribedil, (1995) demonstrated that a 10-minute infusion of 1mM of free drug reduced the compound action potential (CAP) amplitude after a continuous 130 dB SPL pure tone noise exposure (6 kHz, 15 min) without impacting the cochlear microphonic (hair cell) potential. This finding by d'Aldin indicates that the treatment provided improved IHC-AN connection survival following insult without significantly impacting hair cell survival (1995).

4.1.2 Drug Delivery: Local vs. Systemic

The use of local drug delivery to the inner ear is more desirable than systemic approaches primarily due to cochlea features such as the labyrinth-blood barrier, which has a similar function as the blood-brain barrier and limits the passage of molecules from the blood circulation to the cochlear compartment. As compared to systemic delivery, local delivery also reduces the potential for "side effects" of the drugs acting on other systems, enables drugs to remain relatively unaltered because of negligible travel time to

the site of interest, and provides more control over concentration at the site of interest. Therefore, one of the goals of this project is to develop clinically relevant and effective local delivery systems for therapeutic agents into cochlear fluids. Local delivery of pharmaceuticals from a carrier is preferred over direct injection of drugs into the cochlea. The rationale for this preference is the desire to have an increased concentration of the pharmaceutical agent available to cochlear cells for an extended period of time. Therapeutic agents injected directly into the cochlea have short half-lives—minutes to hours—resulting in decreased availability and primary accumulation of the drug near the basal turn of the scala tympani, the common site of injection. Drug delivery interventions utilizing continuous pharmaceutical release results in greater drug accumulation in the cochlea and increased distribution along the cochlear spiral (S. K. Plontke, et al, 2008). Therefore, sustained delivery from days to weeks will be necessary to reach maximum therapeutic benefit and this outcome cannot be achieved by a single injection during cochlear implantation. Sustained delivery of neurotrophic factors has previously been achieved through the use of microcannulas and mini-osmotic pumps. The pumps are capable of dispensing solutions at a programmed rate with one of the primary constraints on the duration of use being the volume of the pump. (Miller, 2007) delivered brain derived neurotrophic factor (BDNF) and fibroblast growth factor 1 (FGF1) for 26 days to the cochlea of deafened guinea pigs via the use of an osmotic minipump and a cannula inserted into the scala tympani. The authors were able to demonstrate that continuous combined factor delivery resulted in increased maintenance of spiral ganglion neurons and their associated peripheral processes.

The locations of the minipump and cannula are a cause for concern. The minipump was implanted in a subcutaneous pocket beneath the scapulae and the cannula was affixed to the dorsal skull using methyl methacrylate (Miller, 2007). Though this is an acceptable delivery method in the lab environment, translation of such a strategy to the clinic would be difficult since the components are not biodegradable and would ultimately need to be removed, at minimum requiring minor surgery. In order to eliminate this concern, there are two delivery approaches that can be used singlely or combined that are not dependent on the prolonged use of osmotic micropumps and cannulas to enable continuous drug administration. One approach is to deliver drugs from biodegradable microparticles placed into the fluids prior to placement of the prostheses. The other is to deliver therapeutics from biodegradable microparticles attached to the surface of the silicone housing used to encase cochlear implant electrodes.

4.1.3 Drug Delivery and Release Assessment

A common target of drug delivery to the cochlea is the perilymph of the scala tympani, making this compartment a common sampling site for determination of pharmaceutical concentration in the cochlea. The perilymph is in contact with the basolateral membrane of the scala media and exchange of fluids between the scala tympani and the scala media where the organ of Corti is located occurs. Two important parameters that must be considered when quantification of intracochlear drug release is desired include the sampling method and the volume of fluid available for sampling. The scala tympani, however, is also in contact with the cochlear aqueduct, which leads to the brain and contains cerebrospinal fluid. This cerebrospinal fluid can “leak” into the cochlea during aspiration of perilymph; this dilutes the sample, possibly contaminates the

sample and lowers the measurable concentration of the released therapeutic. In previous studies that measured drug concentration in perilymph, the vast majority obtained the sample from the scala tympani of the basal turn by perforating the round window membrane or otic capsule (Arnold et al., 2005; Bird et al., 2011; Shayanne A. Lajud & Samudra Sanyal, 2013). Many of these studies have also documented almost immediate and considerable leakage of cerebrospinal fluid into the scala tympani under these conditions. More recently a sampling procedure whereby perilymph is taken from the apical portion of the scala tympani, which is located at a greater distance from the cochlear aqueduct, has been developed and enables the collection of higher purity perilymph samples (A. N. Salt et al., 2012). Furthermore, there are only 10 µL total fluid (perilymph and endolymph) in the guinea pig cochlea. Of this volume, only 4.7µL is from the scala tympani. Even employing the apical sampling method, only the first 2 µL collected is considered to contain pure perilymph (perilymph without any cerebrospinal fluid). Therefore, complete elimination of CSF is not possible if the entire fluid volume of the cochlea is required for analysis; however, a correction factor can be employed to account for this dilution if apical sampling is performed. The minute amount of fluid in the cochlea generally requires that the entire fluid volume of the cochlea be collected in order to enable assessment by liquid chromatography-mass spectrometry (LC-MS) or enzyme-linked immunoabsorbent assay (ELISA), as these techniques have minimum volume requirements because the initial volume is further diluted during analysis.

4.1.4 Rationale for Drug Co-Release in the Cochlea

Because the target population for this intervention are cochlear implantees, it should be noted that there are often multiple factors that contribute to the success of the device in

patient outcomes post-surgery. Some of these factors include closeness of the electrodes to auditory neurons, inhibition of scar tissue formation around electrodes, and inhibition of bone ingrowth/ossification of the scala tympani.(Stöver & Lenarz, 2009). Several options for pharmaceutical intervention in inner ear pathologies have been explored, including anti-oxidants, corticoids/anti-inflammatory agents, and anti-excitotoxic agents. Anti-excitotoxic agents have received the least attention despite the opportunity for synergistic benefit provided by treating hair cells and auditory nerve, both of which serve as crucial parts of the inner ear hearing cascade, and thus were selected for this study wherein the *in vitro* release profile of particles loaded with a neuroprotective agent and drug concentration in perilymph following *in vivo* infusion of agent loaded microparticles will be determined. The hypotheses addressed were:

Hypothesis 1: Microparticles will be able to incorporate and sequester a relevant agent within a particular compartment. This hypothesis tested through the use confocal imaging to visualize the colocalization of the inherent fluorescence of the pharmaceutical agent with that of the polymer dye used to specify a specific microparticle compartment.

Hypothesis 2: Microparticles will be able to release the agent in a sustained and controlled manner *in vitro*. This hypothesis was investigated by monitoring the release of an inherently fluorescent pharmaceutical from the microparticles into aqueous media over a two week time period using ultraviolet spectrophotometry.

Hypothesis 3: Microparticle release of the agent will be detectable in the perilymph of animals that received *in vivo* microparticle infusions. This hypothesis was evaluated through the use of liquid chromatography mass spectrometry (LC-MS) to

assess the amount of drug released from infused microparticles into the perilymph of a guinea pig cochlea.

4.2 METHODS

4.2.1 Particle Fabrication

The following study uses particles made of poly-lactic-glycolic acid (PLGA) or poly-lactic-glycolic acid and dextran acetal (PLGA/dex). A light-emitting polymer, Poly[(m-phenylenevinylene)-alt-(2,5-dihexyloxy-p-phenylenevinylene (MEHPV), was also incorporated into the PLGA and PLGA/dex compartments to facilitate particle visualization via confocal microscopy. PLGA (MW: 44 kDa) with a lactic to glycolic acid ratio of 1:1 was used and PLGA/dex compartments contained 25% PLGA and 75% Dextran Acetal. Electrohydrodynamic co-jetting was utilized to fabricate all particles. Particles containing PLGA/dex compartments were also incubated in an acidic solution (pH=5) for 15 hours to facilitate pore formation on the surfaces of those compartments. Particles were then washed with PBS+1% Tween 20 5 times to remove all acid, then filtered through 10 μm filter. After filtration, particles were centrifuged down and the PBS removed. Prior to an experiment, a known mass of particles was suspended in artificial perilymph (AP; 118 mMNaCl, 30 mMKCl, 2.0 mM $MgSO_4$, 1.2 mM $CaCl_2$, 5.0 mM HEPES; pH = 7.35-7.40, osmolality = 285–294/300mOsm) or artificial perilymph with 55% guinea pig serum albumin (GPSA) to create a 15mg/mL concentration of particles for infusion.

4.2.2 *In Vitro* Piribedil Loading and Release

The PLGA-Dex particles were fabricated as discussed previously with one important exception. Piribedil (Ontario Chemicals) was incorporated into the PLGA at 2% w/w

(drug to polymer in one compartment) prior to jetting. After jetting, Piribedil- loaded particles as seen in **Figure 4-3** were lyophilized and stored until preparation for use. The same preparation protocol used for unloaded PLGA-Dex particles was then followed. For release studies, 0.4 milligrams of Piribedil-loaded particles were incubated inside a permeable membrane exposed to 40 milliliters of phosphate buffered saline (PBS)+1% Tween at 37°C for a two-week period. Samples for drug content assessment were performed in triplicate and collected by removing 1 milliliter volumes from the solution at various time intervals. The concentration of drug in the samples was determined using a UV spectrophotometer to measure absorbance between wavelengths of 200nm and 300nm. Standards were created from serial dilutions of a mass of 5 milligrams of particles suspended in 20 milliliters of phosphate buffered saline (PBS). A standard calibration curve to enable the detection of unknown concentrations of Piribedil for the following concentrations was made and assessed: .0025 mg/mL, .005 mg/mL, .0125 mg/mL, .0375 mg/mL, and .05 mg/mL. The linearity was maintained between 0 and 0.2 millimolar and the correlation coefficients were greater than .991. The total amount of drug released was calculated for each time point. The release profile of each particle was expressed as a percentage according to **Equation 1** as follows:

$$\%Release = \frac{total\ amount\ of\ drug\ released}{total\ drug\ content} x100 \text{ (Eqn. 1)}$$

The values obtained were plotted against time to enable characterization of the phases of particle drug release, from burst release to sustained release and plateau.

4.2.3 *In Vivo* Infusion

For *in vivo* infusions, Hartley guinea pigs (Charles River Laboratory, Wilmington, MA) were anesthetized and the cochleae were accessed as detailed in section **3.2.3**. A 5µL bolus volume of the Piribedil loaded particles was delivered at a rate of 1 µL/minute. The cannula and syringe pump used to facilitate delivery were the same as those described in section **3.2.3**.

4.2.4 *In Vivo* Perilymph Sampling

Sampling of the perilymph for pharmaceutical agents as described by Salt et.al and seen in **Figure 4-4** was undertaken just prior to animal termination. Guinea pigs were anesthetized with xylazine (10 mg/kg intramuscularly) and ketamine (40 mg/kg intramuscularly). The apex of the cochlea was visualized by a ventrolateral approach and the top two apical turns are cleared of mucosa. Once the apex is dry, a thin layer of cyanoacrylate adhesive (vet bond) was placed over the area of the apex designated for perforation. Then a "cup" was formed by adding several layers of silicone (Kwik-Cast) to the outer edges of this area, leaving approximately a 100µm central portion with only the thin layer of cyanoacrylate covering it. In this region, a surgical pick was used to create a small fenestra and escaping perilymph was collected using a microcapillary tube (Drummond, P1549). A total of up to 10µL of perilymph was collected from each animal. In instances where less than 10µL were collected a micrometer was used to measure the length of the fluid column within the microcapillary and a conversion factor of 0.24µL/mm based on the total length (41mm) of the microcapillary was used to determine sample volume. After collection, the perilymph was dispensed from the microcapillary into an eppendorf tube, centrifuged for 2 minutes, and flash frozen in liquid nitrogen. Samples were sealed with parafilm and stored at -80°C until assayed.

Following sampling, animals were euthanized by cardiac puncture and cochleae were removed.

4.2.5 Analysis of *In Vivo* Microparticle Drug Release

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed to determine the Piribedil concentration in perilymph samples. The use of LC allows separation of the pharmaceuticals of interest from a biologically relevant background matrix (native or artificial perilymph) and mass spectrometry enables detection at very low concentrations. The extraction solvent contained 997.5 µL acetonitrile + 2.5 µL Acar mix. Six standards were made with increasing nanomolar concentrations of drug from 0-3000 nanomolars. Standards were prepared by spiking native or artificial perilymph with 3µM stock Piribedil solutions that had been diluted with the extraction solvent. Experimental samples obtained from animals exposed to Piribedil-loaded microparticles were mixed with extraction solvent and vortexed followed by 5-minute incubation at 4°C. The vortex/cooling cycle was completed a total of two times prior to centrifugation of the samples at 15,000 rpm and 4°C for 5 minutes. The sample supernatants were transferred to autosampler vials with inserts for LC-MS analysis. The instrumentation used for analysis consisted of an Agilent 1200 RRLC coupled to an Agilent 6410 Triple Quad LC/MS. Analyte peaks were resolved with the use of a Waters Xbridge C18 (50 mm x 2.1mm, particle size 2.5µm) column.

The liquid chromatographic conditions were as follows: mobile phases: A = 5mM ammonium acetate, adjusted to pH 9.9 with ammonium hydroxide; B = acetonitrile; flow rate: 0.25 mL/min; and injection volume: 2 µL. The gradient began at 25% B and followed a linear regime from 25% to 75%B over 5 minutes before increasing to and

holding 100% for 3 minutes. Then it was returned to 50%B and re-equilibrated for 4 minutes thereby resulting in a total run time of 12 minutes/injection. The mass spectrometry source conditions were: gas temp: 325° C, gas flow: 10 L/min, nebulizing pressure: 40 psi, and capillary voltage: 4000V. All mass spectrometry readings were collected in positive ion mode.

Though the total volume of perilymph in the cochlea is 10µL, the volume of perilymph in the scala tympani, the chamber into which the particle solution is infused, is only 4.7µL (Thorne M et al., 1999). Cochlear fluid space dimensions for six species derived from reconstructions of three-dimensional magnetic resonance images. The balance of the remaining fluid is from the cerebrospinal fluid therefore a simple correction factor would be derived from **Equation 2** as follows:

$$Correction\_factor : \frac{volume\_of\_perilymph\_collected}{volume\_of\_perilymph\_in\_scala\_tympani} \quad \text{(Eqn. 2)}$$

4.3 RESULTS & DISCUSSION

4.3.1 Incorporation of Piribedil into Microparticles and Sequestration

Piribedil has poor solubility (less than 0.1 mg/mL) in aqueous solution (M. Demirel R. H. Müller, 2001), however, it also has an inherent fluorescence detectable by UV spectrophotometry as well as confocal lasers. The aforementioned fluorescence enables Piribedil to be visualized within the polymer matrix of the microparticle after fabrication by excitation and emission in the red wavelength range. In the dextran/polylactic glycolic acid (PLGA/Dex) particles, Piribedil was only incorporated into the PLGA, therefore it should only have been visible within the PLGA compartments of the microparticle. Upon examination of Piribedil-loaded particles using confocal laser scanning microscopy

(CLSM), the sequestration of the Piribedil into the PLGA-only compartment was confirmed, as seen in **Figure 4-5.** The PLGA-only compartment appeared green due to the incorporation of MEHPV into the polymer matrix, and the PLGA/Dex compartment appeared blue due the incorporation of PTDPV, thus enabling visualization of the separate compartments post-jetting. Furthermore, overlay of excitation of the microparticle within the red channel resulted in the PLGA-only compartment appearing yellow due to the overlap of the green fluorescence from the polymer and the red fluorescence from the Piribedil. No change in appearance occurred in the PLGA/dex compartment when an overlay of excitation of the microparticle within the red channel is made (such as the appearance of a purple color, which further allows confirmation of the sequestration of Piribedil following microparticle fabrication.)

4.3.2 *In Vitro* Release of Piribedil from Microparticles

The visualization of the Piribedil with CLSM confirmed the presence of the pharmaceutical within the microparticle and enabled release studies to be conducted to characterize *in vitro* release profiles and *in vivo* cochlear fluid concentrations following infusion of drug loaded microparticles. The *in vitro* release profile of the Piribedil from monoporous and biporous multicompartmental particles is shown in **Figure 4-6**. The initial release from the microparticles in the first 24 hours following incubation was around 20%. A sustained release profile was evident between sampling days 2 and 9 before particle release plateaus around day 14. Over a 14-day time period, the high density monoporous and high density biporous particles averaged a cumulative release of their initial drug content of 69.2% and 71.3%, respectively. There was no significant difference in release between monoporous and biporous particles either in profile or

cumulative totals. This result provided additional confirmation of Piribedil sequestration in the PLGA-only compartment because that compartment is the same in both particle types. The only way that a difference in release could be observed would be if the drug had leached into the PLGA/dextran compartment where the porosity differences could then have an impact. If that were the case, the biporous particles would be expected to have a faster release profile since pores increase the available surface area for interaction between the polymer, which degrades by hydrolysis, with the aqueous environment.

4.3.3 *In Vivo* Perilymphatic Piribedil Concentration

Based on the *in vitro* particle release profiles and consideration of the time to achieve therapeutic release of Piribedil, the 7-day post infusion timepoint was selected to perform perilymph sampling to ascertain *in vivo* concentration of Piribedil. Initially, collection of perilymph samples was attempted in a total of 6 animals; however, due to technical difficulties encountered during collection, only 4 samples could be used for analysis. The total volume of fluid collected ranged from 5-10.2 µL. Piribedil was detected in all analyzed samples. The aforementioned variability in sample fluid volume also correlated with variability in quantified Piribedil concentration as seen in **Table 4-1**. Two of the collected samples, NAN 30 and NAN 32, had volumes allowing the assumption that most, if not all, of the approximately 2 µL of pure perilymph was captured. These samples were within therapeutic range both before and after applying the correction factor. Therefore, a minimum collection volume was imposed upon the additional samples collected in order to measure *in vivo* Piribedil concentration. In the subsequent round of collection, 4 additional samples met the volume criteria and were assessed for Piribedil concentration. These samples all had both measurable and therapeutic levels of

Piribedil. Though the institution of a minimum volume did lead to decreased variability in the concentration of Piribedil in the perilymph, some of the variability that remains in the apparent perilymph concentration could be the result of the variability in the number of particles that persist in the cochlea post infusion. Therefore, one approach to improve the amount and consistency of Piribedil available 7 days post infusion would be to incorporate targeting ligands for cell populations in the cochlea such as the epithelium. These ligands could be attached to particles, thereby increasing the number of particles retained in the cochlea and subsequently the amount of drug available for release. Perhaps more importantly the remaining particles would be retained at therapeutic target sites, thus likely increasing efficacy. Alternatively, the surface of the particles could be modified to enable attachment to the cochlear implant itself. This approach would also provide increased control over particle number and persistence within the cochlea.

4.4 CONCLUSION

Piribedil was successfully incorporated into the PLGA-only compartment of PLGA/dextran particles. *In vitro* assessment provided confirmation of the sequestration of Piribedil because no difference was observed in the release profile of biporous particles when compared to monoporous particles. Further, the controlled and sustained release of Piribedil from the multicompartmental microparticles was demonstrated. *In vivo* assessment of perilymph from animals infused with Piribedil-loaded particles demonstrated that the microparticles could release detectable amounts of drug for at least 7 days. In addition, the amount of Piribedil detected in cochlear fluids at the aforementioned timepoint was capable of reaching therapeutic levels. Controlled and

measurable release of drug from the multicompartmental microparticles is possible and represents a potential intracochlear drug delivery platform.

4.5 FIGURES & TABLES

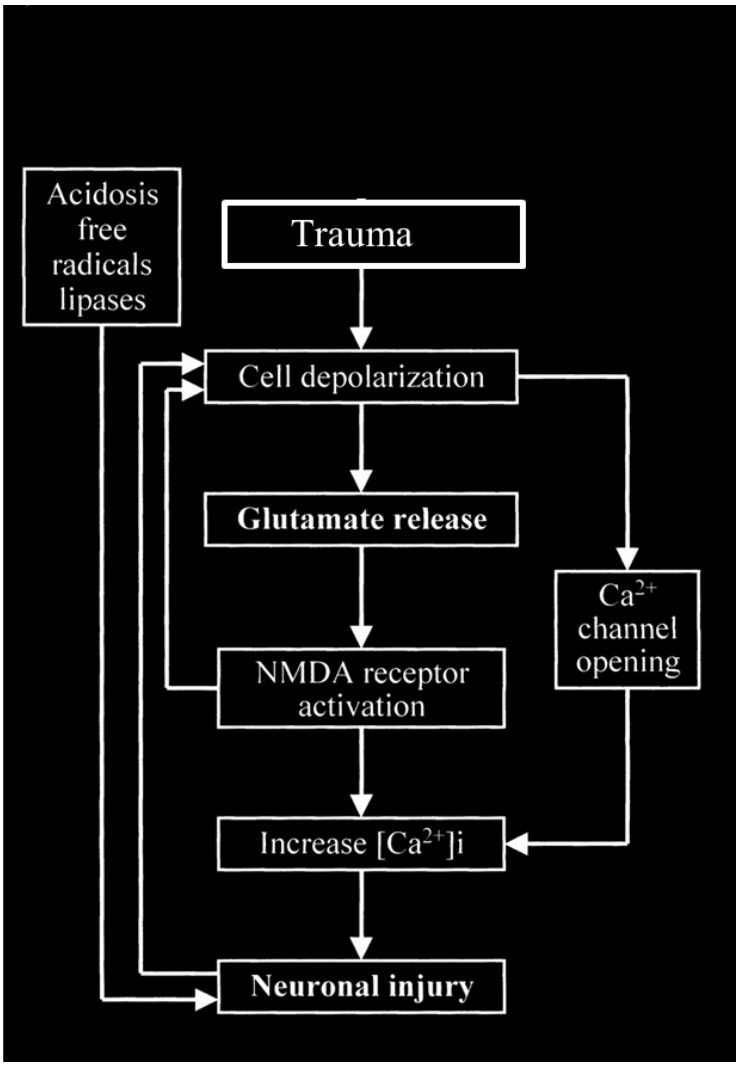


Figure 4-1. Excitotoxicity cascade indicating the chain of events caused by cochlear trauma that can lead to excitotoxicity and loss of inner hair cell auditory nerve synapses. Adapted from (Mark et al., 2001).

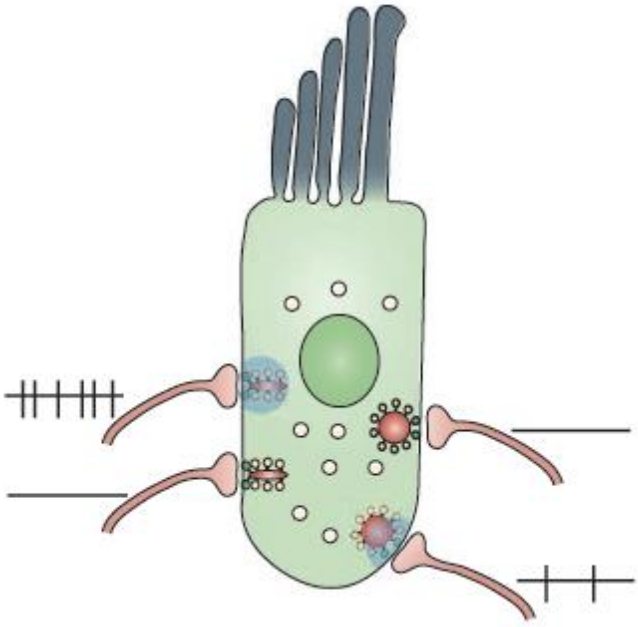
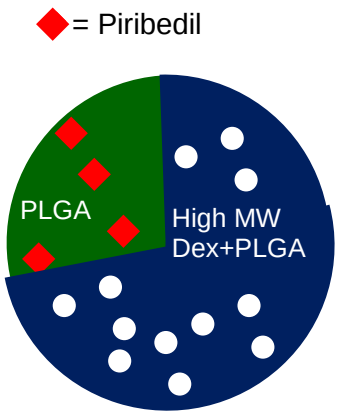


Figure 4-2. Image illustrating the site of connection between the inner hair cell and auditory neuronal processes. Trauma such as noise and potentially cochlear implantation may lead to the degeneration of these connections even when the inner hair cell is still present (Matthews & Fuchs, 2010).

A



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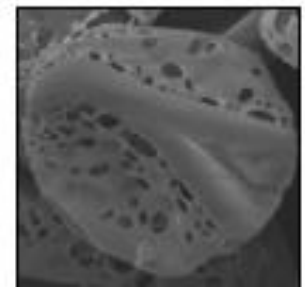


Figure 4-3. A) Schematic of Piribedil-loaded biporous particle. PLGA/Dex compartment (blue) and separate PLGA compartment (green) wherein Piribedil (red) is incorporated into the PLGA-only compartment. White circles represent pores. B) Scanning electron microscopy (SEM) of particle Courtesy of Sahar Rahmani (Rahmani et al., 2013).

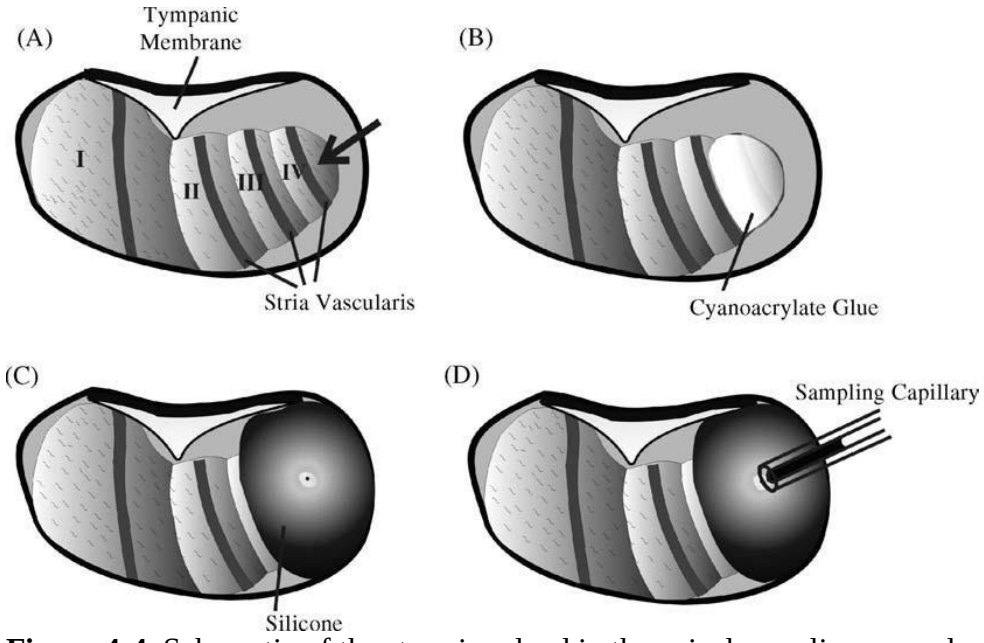


Figure 4-4. Schematic of the steps involved in the apical sampling procedure: (A) Use ventral approach to expose cochlea and remove mucosa from apical turns; (B) Apply a thin layer of cyanoacrylate glue to the bone over apical turns; (C) Construct a cup using two-part silicone around the cochlear apex. Apply silicone very thinly to the region to be perforated. Once ready to sample, perforate the apex with a fine pick; (D) Collect all fluid efflux from the apical perforation site into a calibrated capillary tube (Alec N Salt et al., 2007).

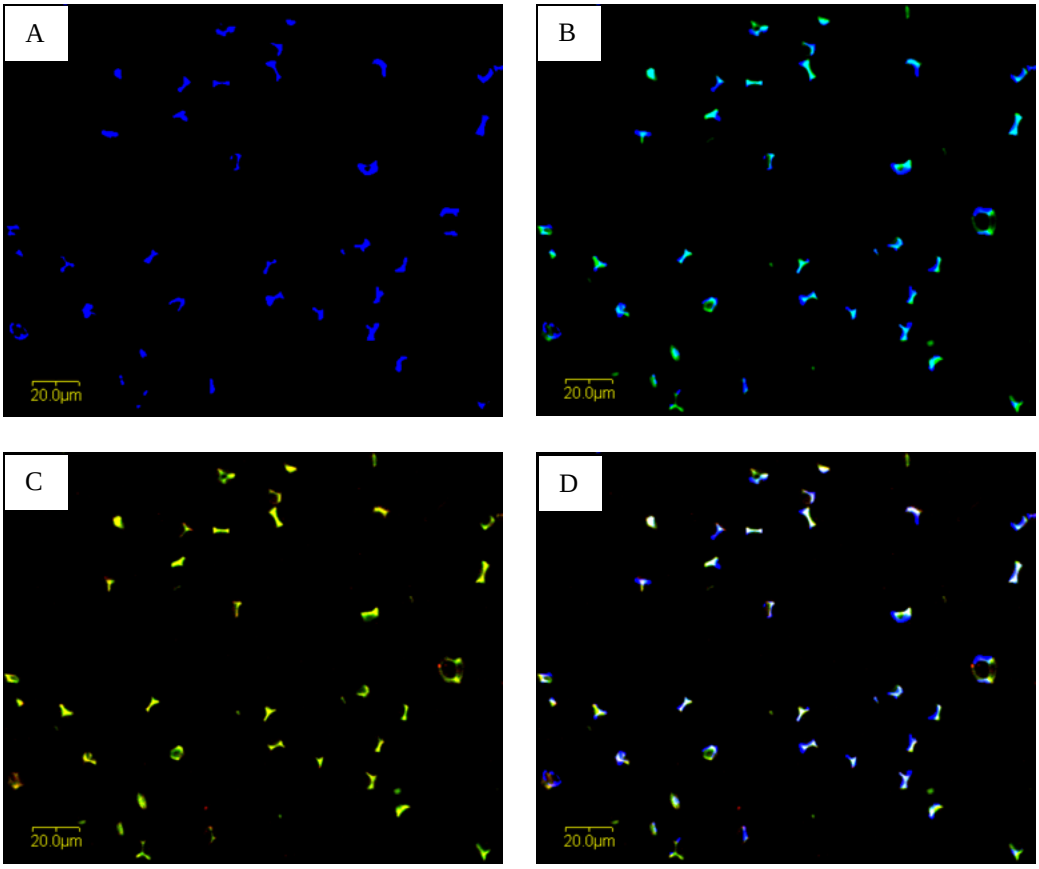


Figure 4-5. Biporous MPs loaded with Piribedil A) PLGA/Dex.(blue) compartment B) Distinct PLGA (green) and PLGA/Dex.(blue) compartments C) Piribedil in PLGA (yellow) D) All compartments .

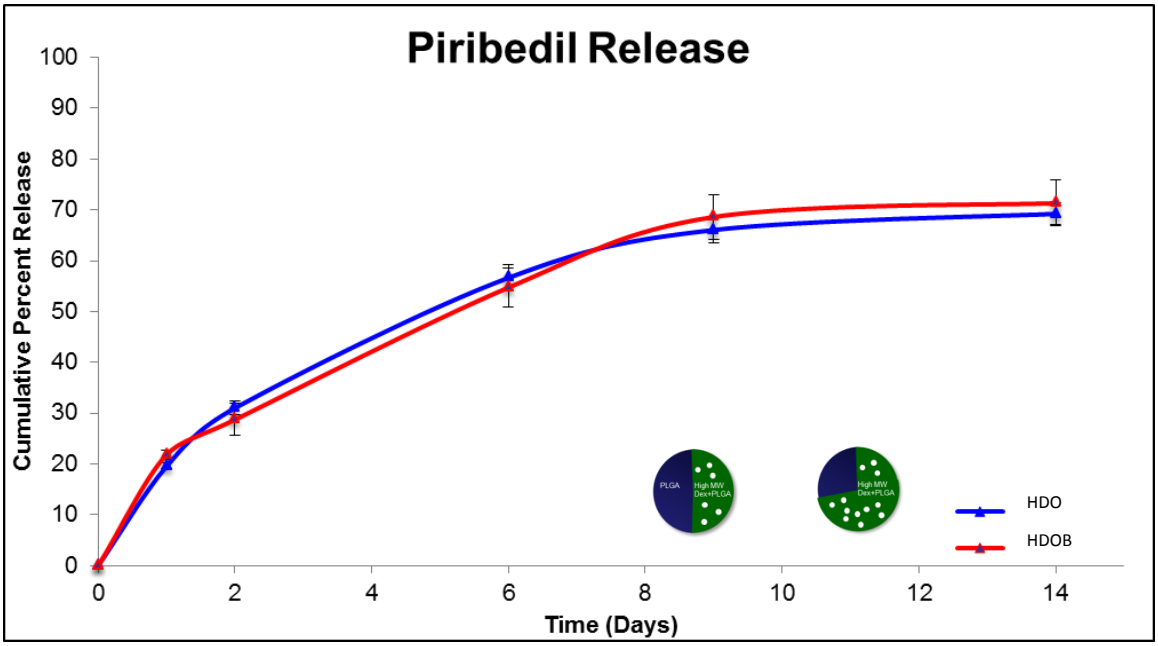


Figure 4-6. *In vitro* release profile of Piribedil-loaded monoporous (HDO) and biporous (HDOB) microparticles.

Table 4.1. Perilymph Volumes and Piribedil Concentrations from *in vivo* Sampling

Specimen	Volume (µL)	Concentration (nM)	Corrected Concentration (nM)
NAN 30	10.2	339.23*	736.20*
NAN 32	9	106.04*	207.57*
NAN 41	8.4	207.41*	370.68*
NAN 44	7.22	205.73*	316.04*
NAN 45	7.1	192.01*	290.06*
NAN 34	6.88	223.43*	327.07*
NAN 31	6	13.68	17.47
NAN 29	5	44.48	47.32
Average	7.45	166.50	289.05
Standard Deviation	1.67	106.13	222.84

* indicates that therapeutic level was attained

Chapter 5

Conclusions & Future Directions

5.1 CONCLUSIONS

This dissertation characterizes multicompartmental microparticles and their use as vehicles for inner ear drug delivery. This work provides the first evaluation of multi-release particles for local drug release in the cochlea for both *in vitro* and *in vivo* environments; it defines the hurdles to efficacious use of these drug carriers for modifying inner ear function; and it identifies a research pathway that may enable clinical translation of these drug carriers in the efficacious treatment of inner ear disease.

In **Chapter 2**, we ascertained preferred design and infusion parameters for local drug delivery in the cochlea. Parameters were classified as preferred if their modulation led to an increase in the visualization of the microparticles within cochlear tissues. Multicompartmental microparticles fabricated via electrohydrodynamic co-jetting were evaluated in *ex-vivo*, *in vitro*, and *in vivo* systems. The identified preferred parameters from *ex-vivo* and *in vivo* assessment were associated with particle design, particle infusion protocol, or tissue processing and post harvesting. In particular, for particle design, the fluorescence intensity of particles was improved by increasing the amount of polymer dye incorporated during particle fabrication. The most important parameter of the infusion protocol was the composition of the background solution used to suspend the particles to facilitate delivery. Finally tissue processing procedures such as the use of passive fixation rather than vascular perfusion, elimination of slide rinsing, and use of a

commercial agent with decreased time to decalcification also improved visualization of microparticles following infusion. *In vitro* infusion experiments revealed that microparticle porosity did not impact the distribution of microparticles within a cochlear-like microchannel.

In **Chapter 3**, *in vivo* microparticle persistence, distribution, and impact on cochlear function and histopathology were assessed. Confocal microscopy of cochlear cross sections demonstrated the presence of microparticles at 1 and at least 7 days post infusion. Microparticles were located primarily in the basal and second turns. Neither particle distribution nor number changed with increased time indicating that if particles avoided clearance in the first 24 hours following infusion, they persisted for functionally and therapeutically relevant durations. Further, stereological analysis was used to estimate the total number of microparticles present at 7 days post infusion throughout the entire cochlea which provides baseline for assessing future modifications aimed at increasing particle persistence post infusion. This development also informs aids understanding of dosing requirements pharmaceuticals that could be used based on percent drug incorporation into particles and particle persistence following infusion. Microparticles were not detected in the contralateral ear at 1 or 7 days. Functional analysis with auditory brainstem response demonstrated that infusion of blank particles could be delivered with no negative impact the hearing and no effect on hair cells. Analysis of variance assessment (ANOVA) of hair cell counts revealed that the number of hair cells present in the first turn of treated cochleae were similar to those of non-treated cochleae. Interestingly, the number of hair cells present in infused cochlea were significantly different than those in non-infused cochlea in the third and fourth turns

despite minimal particle presence. Importantly, microparticle infusion did not induce an immune response as indicated by the comparable numbers of CD45 positive (white blood) cells present in infused cochleae when compared to non-infused contralateral cochleae.

In **Chapter 4,** pharmaceutical release from the multicompartmental microparticles was evaluated both *in vitro* and *in vivo*. *In vitro* release of Piribedil, an anti-excitotoxic agent, was evaluated by UV-spectrophotometry and demonstrated that the microparticles were capable of sustained release on the order of weeks which would be suitable for intracochlear drug delivery. Liquid chromatography mass spectrometry (LC-MS) analysis of perilymph obtained 7 days after infusion of Piribedil loaded particles identified the pharmaceutical at detectable levels in all samples. For the first time, we demonstrated the release of therapeutic levels of a pharmaceutical from an intracochlear particle system. Prior to assessment of *in vivo* drug concentration, the incorporation and sequestration of Piribedil into the appropriate microparticle compartment was confirmed via confocal laser scanning microscopy. In addition, the porosity of particle compartments did not impact the sustained release profile *in vitro*.

Multicompartmental particles fabricated by electrohydrodynamic co-jetting had not previously been utilized in biological tissues or an animal model. Therefore this research has resulted in the refinement of an innovative platform for use *in vivo* with great potential for expanding the selectivity and utility of cochlear drug delivery. Since porosity did not induce differential particle distribution, future efforts should focus on particle shape, rather than porosity, as a design feature with the potential to impact distribution of free particles during infusion. Additionally, this research established the

Piribedil release profile from MPs *in vitro*, and in the future it could be informative to assess *in vivo* perilymph samples at various timepoints to determine how well the *in vivo* profile correlates with that obtained *in vitro*. Although, Piribedil was used as the test article for this work, other anti-excitotoxic agents could be used. Further these agents could target other facets of excitotoxicity beyond cell responsiveness to glutamate such as the rate of glutamate re-uptake (recycling) or glutamate production. This would aid in the determination of the most efficacious component of the excitotoxicity cascade to target in reduction efforts.

5.2 FUTURE DIRECTIONS

5.2.1 Drug Delivery in the Cochlea

The use of pharmaceutical intervention to ameliorate cochlear pathologies and/or their associated symptoms has been in place for several decades, however, with notable gains in technology, particularly the fabrication of micro- and nanoparticles, the possibilities for local drug delivery to the cochlea are expansive. Use of micro- and nanoparticles is also capable of extending the agents used for local treatment to include growth factors, peptides, DNA, and siRNA. Further micro- and nanotechnologies can be used to facilitate monitoring of the cochlea in real time for assessment of characteristics such as cochlear blood flow and/or be utilized as monitors of enzymatic activity. Particle platforms will be an integral part of the future of cochlear drug delivery because they can provide local and targeted delivery that will reduce patient side effects. In addition, particle systems will provide scientists with important information concerning the microenvironment present under pathological conditions in the cochlea.

5.2.2 Assessment of Efficacy of Microparticle Drug Delivery in Noise Trauma Model *In Vivo*

This dissertation described the development and characterization of the multicompartmental microparticles for cochlear drug delivery. The experimental model used for this work was exclusively a normal hearing ear. The next steps in the short term evaluation of the multicompartmental microparticles should investigate the utility of microparticle drug release in a traumatized ear model. Inner ear trauma may be induced by chemical, mechanical, or acoustic stimuli. In future studies an acoustic or "noise" model should be studied as a proof of principal pathological model to determine whether local delivery of anti-excitotoxic pharmaceuticals from the microparticles can provide protection to inner hair cell-auditory connections. Acoustic stimuli are suggested because its use provides an opportunity to induce selective hearing loss (in a particular region). The outline for the experiment may be seen in

Figure 5-1. The experiment involves noise exposure of normal hearing guinea pigs (as determined by auditory brainstem response) 7 days following intrascalar placement of microparticles with or without test pharmaceuticals. Day 7 post infusion was chosen for noise exposure because of the *in vitro* release data for the Piribedil loaded particles which demonstrated that at day 7 sufficient Piribedil had been released and remained likely to provide therapeutic effectiveness. Further, previous research has shown that ABR thresholds of surgical animals have generally recovered so that they are comparable to pre-infusion values by day 7. Animals will be exposed bilaterally to a 122 octave band noise (OBN) for 3 hours. Without intervention, this exposure should cause permanent functional and histological damage, including damage to the afferent peripheral processes of the auditory nerve.

At 5 days post microparticle infusion and 14 days post-noise ABRs will be used to assess hearing to define surgical and noise induced changes from baseline hearing sensitivity. Post-life assessment will include immunohistochemistry of hair cell loss and loss of inner hair cell-auditory nerve (IHC-AN) connections. These evaluations will be performed on cochlea harvested 14 days following noise exposure. We will obtain hair cell counts from the Phalloidin staining and generate cytocochleograms to determine the loss of hair cells and the distribution of the losses in the cochlea based on our noise trauma model. Standard analysis of variance (ANOVA) testing will be used to determine whether significant differences (in the number of synapses present occurs between groups.

Confocal laser scanning microscopy will be used to acquire z-series images of inner hair cells and their associated IHC-AN puncta. The IHCs will be visualized with a Phalloidin (f-actin) stain and the puncta will be labeled with C-terminal Binding Protein 2 (CtBP2) which is a protein that occurs at the presynaptic terminal of the inner hair cells before interfacing with auditory nerve. The z-stacks will be used to provide a quantitative assessment of the number of CTBP2 immunolabeled "puncta" per inner hair cell at 3 pre-determined regions of interest (ROI) along the cochlear spiral as seen in **Figure 5.2** and **Figure 5-3**. The aforementioned regions of interest are categorized by frequency and collected at 0.4, 4, and 20 kilohertz. Frequency values were selected to span the range of the distribution of particles as determined by the distribution studies outlined in chapter 3. Following noise exposure, we would expect, Piribedil infused ears to have increased IHC-AN connections as compared to those ears that received blanks or were untreated. We would not expect differences in hair cell loss between the conditions because

Piribedil is used as an anti-excitotoxic agent in this study and is not known to impact hair cell survival.

A pilot study (n=3) with Piribedil loaded particles was conducted that indicated the following: the chosen noise exposure induced large losses of outer hair cells in both treated and untreated ears as indicated by the cytocochleograms in **Figure 5-4**. These findings correlate with functional losses present in the auditory brainstem responses of the pilot animals as shown in **Figure 5-5**. In addition, there were large spikes of inner hair cell loss in several of the ears, both treated and non-treated, which is undesirable. Therefore, the noise exposure parameters will need to be modified to produce a damage profile that does not include losses of inner hair cells. The number of inner hair cell auditory nerve synapses remaining were indicated by CTBP2 staining as seen in **Table 5-1**. The number of CTBP2 positive cells were comparable between treated and untreated ears at lower frequencies although a difference existed at 20 kHz, the higher frequency assessed wherein untreated ears contained increased amounts of CTBP2 staining. However, more animals are needed to implement the full experimental design as seen in **Figure 5-1**. Additionally, in the long term, it would be worthwhile to investigate the efficacy of microparticle release in a cochlear implant trauma model because that would most closely mimic the intended clinical application. The same general experimental outline could be applied, however, the trauma that occurs on day 7 would be implant insertion. Alternatively, the experimental design could be modified such that particle infusion occurs at the same time as implantation. Further, the number of treated animals may need to be increased due to increased variability of damage induced by manual insertion that we have observed in our lab (unpublished results).

5.2.3 Assessment of Growth Factor Release: GDNF

One of the potential benefits of using the chosen polymeric microparticle system for drug delivery is the availability of functional groups allowing surface modification with growth factors and attachment of targeting ligands. Several growth factors have been identified as contributing to the maintenance or regrowth of peripheral nerve processes following trauma, including neurotrophic growth factors such as brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and glial derived neurotrophic factor (GDNF). In the near future, studies should investigate the benefit of providing acute and sustained delivery of a biologically active neurotrophin in addition to an anti-excitotoxic drug such as Piribedil. In particular, GDNF has been shown to promote spiral ganglion cell survival both *in vitro* and *in vivo* in addition to attenuating spiral ganglion neuron (SGN) and hair cell loss after ototoxic insult. Since GDNF is capable of influencing hair cell survival, a microparticle incorporating Piribedil and GDNF could be used to simultaneously facilitate the preservation of hair cells, spiral ganglion neurons, and inner hair cell-auditory nerve connections in cochleae exposed to trauma. Both *in vitro* and *in vivo* assessments should be employed to assess the feasibility and utility of attachment of GDNF onto the microparticles.

Confocal laser scanning microscopy would be used to confirm the attachment of GDNF onto the surface and within the porous cavities of the microparticles. GDNF would be attached to the surface of and within particles because incorporation into multicompartmental particles as fabricated in this dissertation could denature the protein. A similar strategy was used by Tan et al. to release brain derived neurotrophic factor on the order of weeks to months (Tan et al., 2012). Alternatively a core shell particle could

be created to encapsulate the GDNF, albeit utilizing different polymer components. Further, GDNF concentration on the microparticle surface will be quantified with an enzyme-linked immunosorbent assay (ELISA) utilizing antibodies to human GDNF to assess the concentration of GDNF in the incubation solution prior to and following microparticle incubation. In addition, i*n vitro* and *in vivo* release of the GDNF from the microparticles will also be determined by ELISA. For *in vitro* release sturdies, a profile will be developed over a 30 day period. The timepoint for assessment of release *in vivo* would be determined by the time it takes to achieve therapeutic concentrations *in vitro*.

We would expect the release of GDNF from the microparticles to occur primarily by diffusion. Therefore the release profile should contain a small initial "burst" release followed by a steady increase in release until a plateau is reached. This work would help extend the utility and variety of therapeutic approaches that could be investigated using the multicompartmental microparticles as a delivery platform.

5.2.4 Efficacy of Local Delivery from Multi-Agent Loaded Microparticles *In Vivo*: Piribedil/GDNF and Piribedil/Anti-Oxidant

Following characterization of *in vitro* release, *in vivo* efficacy studies could be performed. In addition to hair cell and CTBP2 counts, spiral ganglion counts would also be made to determine whether the release of GDNF increased the density of neurons present within the modiolous and auditory nerve following noise exposure. The density and growth/regrowth of peripheral processes would also be assessed. The addition of the aforementioned assessment would also necessitate an increase in the duration of the experiment from 2 to 8 weeks post noise exposure as degeneration of spiral ganglion

neurons occurs on a slower timescale than the disappearance of the inner hair cell auditory nerve connections.

In addition to the bursting of peripheral processes, cochlear insult can induce the formation of reactive oxygen species (ROS). Though this dissertation, and the future work highlighted previously, have largely focused on preservation of inner hair cell auditory nerve connections, an investigation of combinatorial therapy with the addition of anti-oxidants may provide more efficacy in ameliorating induced pathology than either type of drug alone. This premise is based on the recognition that such a treatment paradigm attacks both pre-synaptic and post-synaptic components of the inner ear hearing cascade, and ROS formation has been found to play a role in excitotoxicity (Mark et al., 2001). Anti-oxidant therapy is an appropriate complimentary treatment strategy because it aids in the prevention of hair cell death which has a secondary impact on the viability of auditory neurons. Of particular interest would be vitamins A, C, and E, anti-oxidants that function as free radical scavengers which help eliminate the reactive oxygen species (ROS) generated by cochlear trauma. In the presence of ROS, hair cells undergo apoptotic cell death which leads to the deafferentation of auditory nerve, subsequent peripheral process degeneration, and eventually spiral ganglion cell death (Le Prell, 2007; Maruyama et al., 2007; Sha, 1999). This outcome is detrimental as the presence of viable spiral ganglion neurons capable of responding to stimulation is crucial to the efficacy of a cochlear implant. While anti-oxidant therapy has been demonstrated to have a positive impact on hair cell survival after insult, it has not been shown to significantly impact IHC-AN connection survival.

Experiments would characterize the pathophysiology of the cochlea following permanent threshold shift (PTS) inducing noise exposure. Efficacy of local delivery of Piribedil/GDNF or Piribedil/ACE loaded microparticles on auditory brainstem responses (ABRs) will be taken to provide a clinically relevant functional measure of both the pathology induced by the insult and assess the effectiveness of these interventions and this model. The utility of the intervention is further assessed by comparison of spiral ganglions (SGNs), hair cells (HCs), and inner hair cell auditory nerve (IHC-AN) synapses in treated and untreated groups.

We anticipate that the addition of local release of GDNF from the microparticles will reduce the loss of HC, IHC-AN connection, and SGNs induced by the noise exposure as compared to Piribedil only particles. We also anticipate that addition of local release of Vitamins A, C, and E will reduce HC losses induced by the noise as compared to both Piribedil only and GDNF/Piribedil particles. These studies would provide insight on whether synergistic effects could occur between treatments of different classes of therapeutics and establish multicompartmental microparticles as a valuable drug delivery vehicle for such studies.

5.2.5 Microparticle Attachment to Cochlear Implant

The use of multicompartmental microparticles for therapeutic cochlear drug delivery is dependent upon the number of particles that persist following perfusion. The work in this dissertation demonstrated that untargeted particles persist in the cochlea, even though the number was a small fraction of the number delivered. To increase particle persistence, particle delivery strategies that do not involve infusion of a free particle suspension should be investigated in the long term as these methods could offer increased control of

particle number and resistance to clearance. Thereby, an extension to the studies described in the main body of this dissertation includes the surface modification of microparticles to enable the attachment of particles to a substrate such as the silicone housing of a cochlear implant. This attachment would limit particle clearance via macrophage uptake and loss to the general circulation or down the cochlear aqueduct.

The microparticles that will be used in this study will be attached to the aforementioned substrates via covalent or click chemistry strategies. Particle orientation and appearance after attachment and the extent of particle coverage of implant housing will be assessed with scanning electron microscopy. Further the strength of particle attachment will be assessed with adhesion testing and confocal laser scanning microscopy. The impact of microparticle attachment on microparticle release would also be investigated *in vitro*. This study would allow increased control of microparticle distribution, including enhanced access to upper turns of the cochlea, and dosing because particles could be uniformly distributed along the implant and the number/concentration of particles immobilized on the surface could be easily determined thereby enabling more precise predictions of dosing as long as the amount of drug loaded within particles was known. In addition, more precise control of dosing could enable agents with higher therapeutic concentrations to be used efficaciously in cochlear drug delivery that would not otherwise be viable options.

The work outlined in this dissertation as well as that proposed or in progress under future directions, enhance the development of strategies for drug delivery to the inner ear. This was accomplished through the evaluation of the first multi-release particle system for local drug release in the cochlea wherein design and infusion parameters for local

drug delivery in the cochlea were identified such that microparticles could be visualized and were able to persist for at least 7 days in cochlear tissue. In addition, for the first time, the release of therapeutic levels of a pharmaceutical from an intracochlear particle system was demonstrated. These accomplishments will serve as a springboard for the design and evaluation of multi-release therapeutic systems which may ultimately maximize the benefit of cochlear implantation for individuals with residual hearing. Benefit will be from the combined use of therapeutically preserved low frequency acoustic hearing and CI enabled high frequency electrical hearing. Specifically implantees should expect to have increased speech discrimination in noise (such as talking in a crowded restaurant) and increased detection of sound pitch (which is required to recognize song instrumentals in the absence of words). This means that one day a young woman will have less anxiety about being at a crowded party and a young man will be able to fully appreciate a symphony.

5.3 FIGURES & TABLES

	n	ABR/IO	Treatment	Noise	ABR/IO	Sacrifice
Days		Pre	days 0-21	Day 7	Day 5/21	Day21
Anti-excitotoxicity group						
Group 1	8	Yes	Unloaded MPs	Yes	Day 5/21	Day 21
Group 2	8	Yes	Piribedil loaded MPs	Yes	Day 5/21	Day 21
Total n	16					

Figure 5-1. Outline of experiment to determine efficacy of local Piribedil delivery in response to trauma such as noise. The duration of each phase of the experiment is indicated as well as the timing of functional assessments.

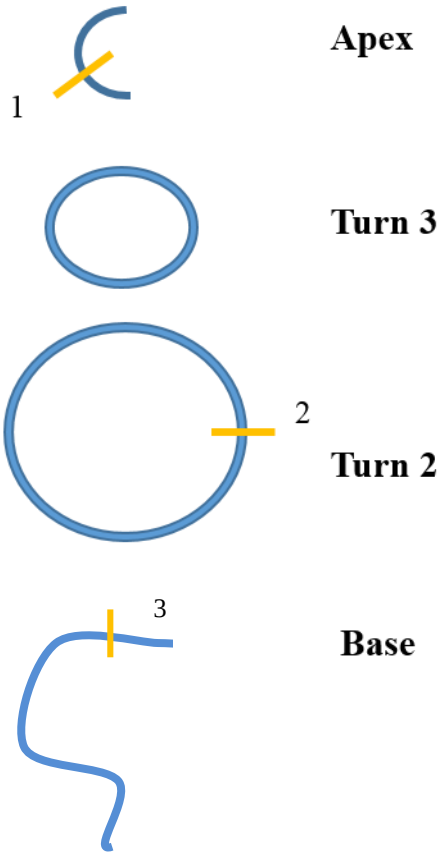


Figure 5-2. Schematic representing inner hair cell-auditory nerve (IHC-AN) regions of interest (ROIs) for C-Terminal Binding Protein Assessment in the guinea pig cochlea. 3 ROIs corresponding to 1:0.4kHz, 2:4khz (noise), and 3:20kHz.

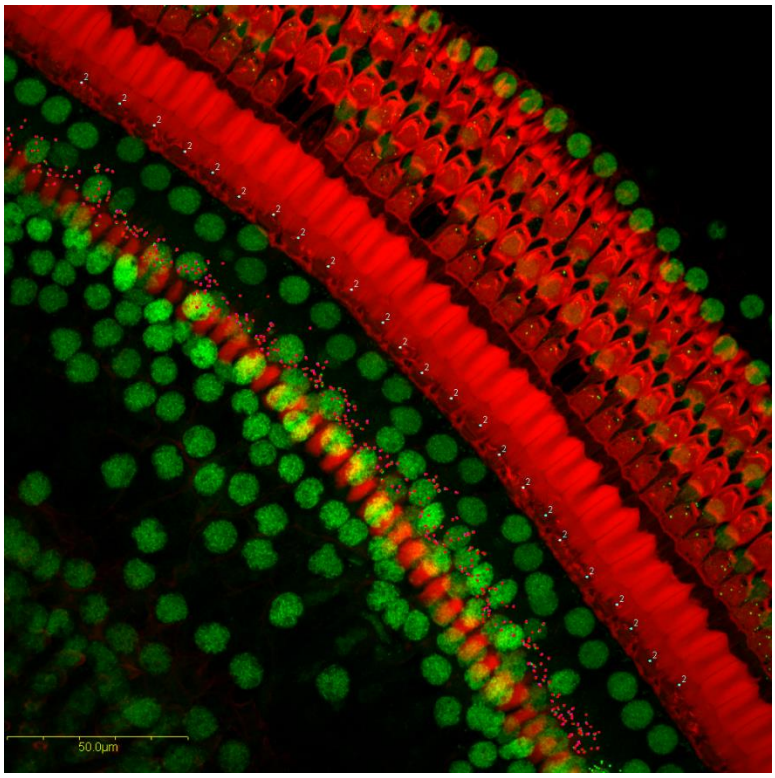
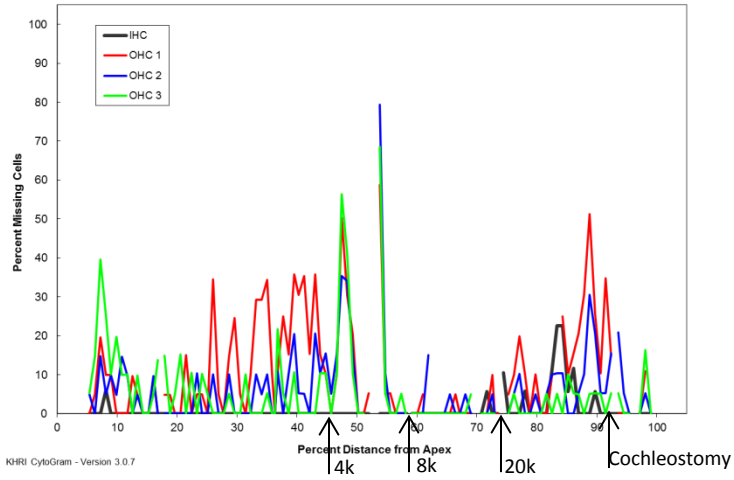
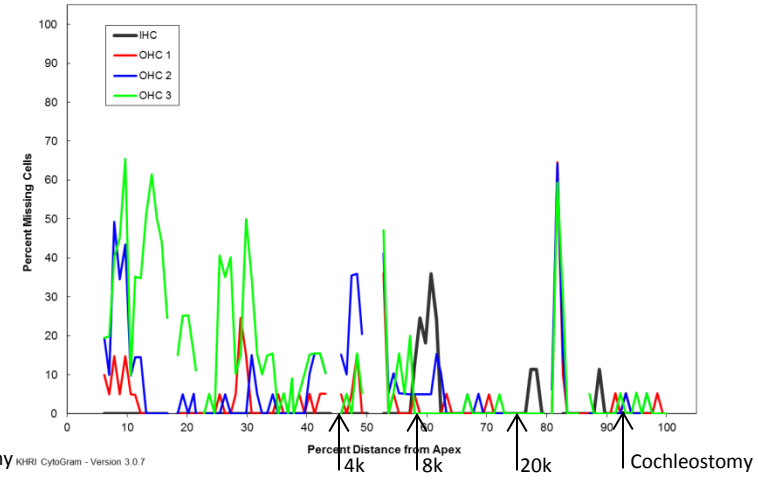


Figure 5-3. CTBP2 z-stack reconstruction demonstrating staining and counting.

NAN 36-L



NAN 36-R



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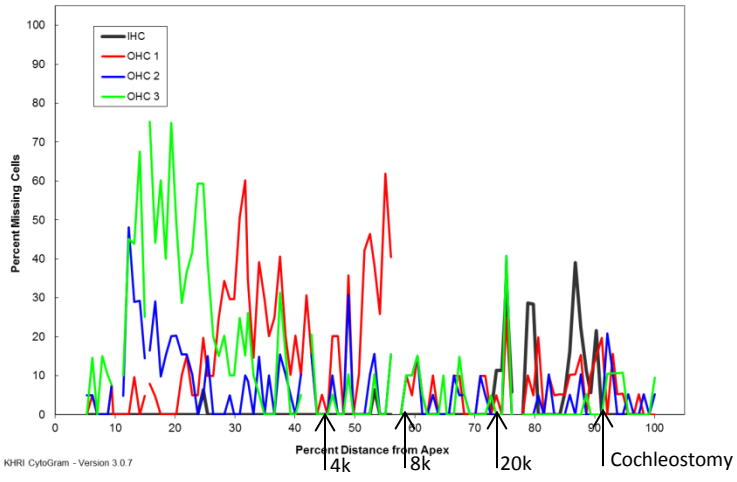
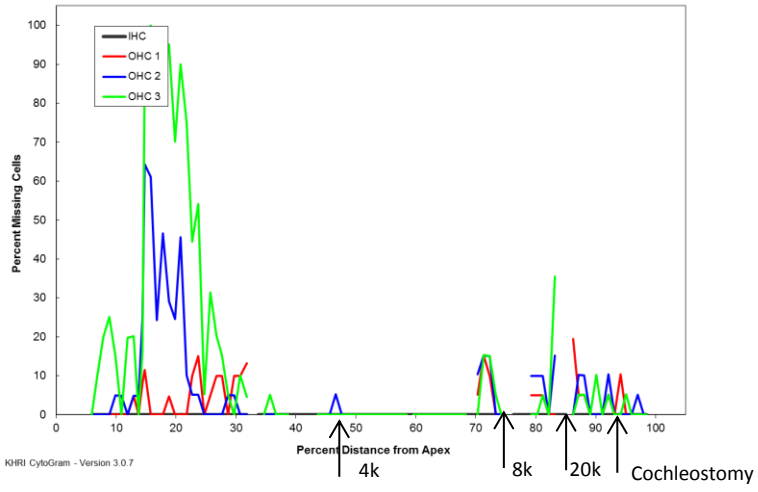


NAN 37-R



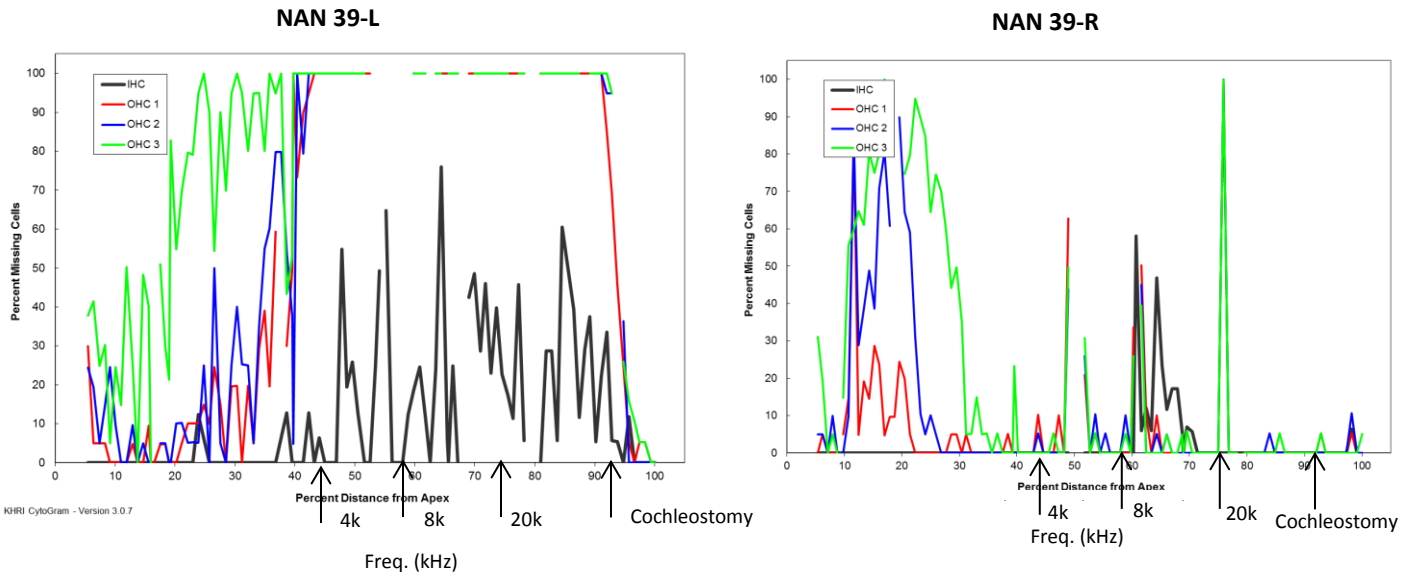
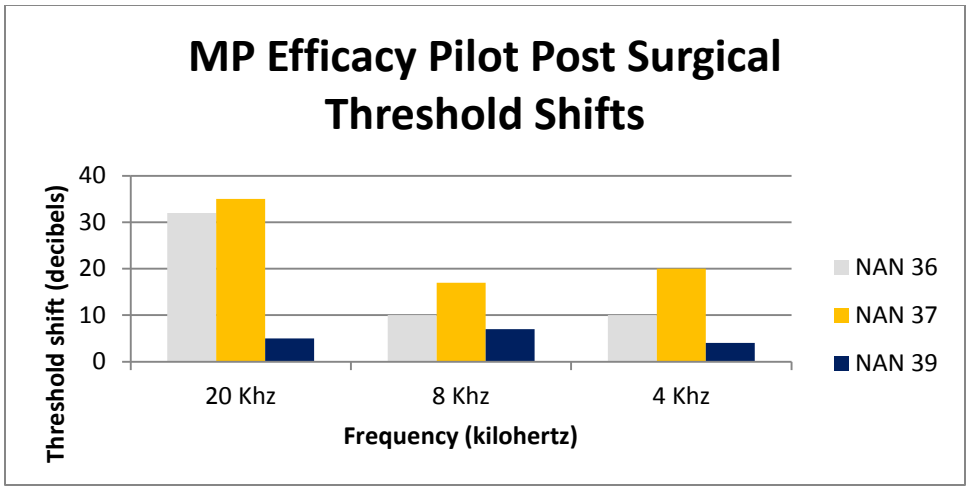
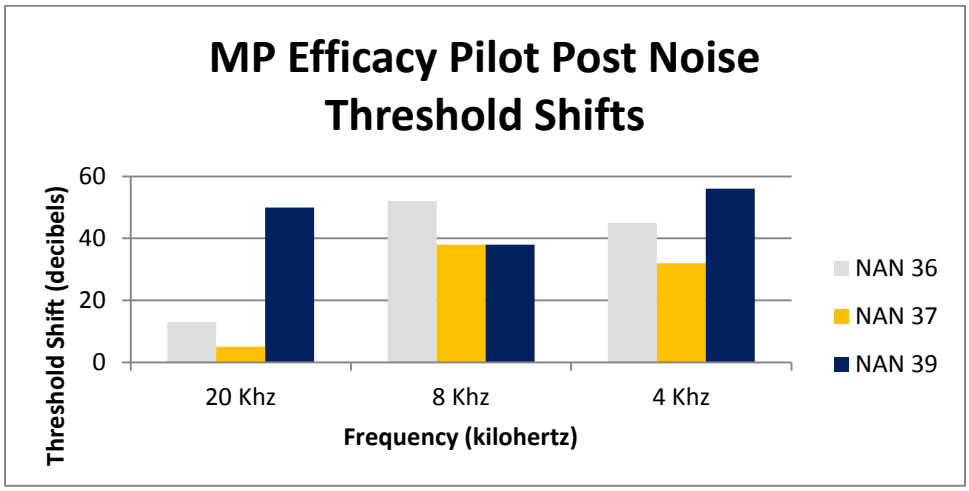


Figure 5-4. Cytochochleograms of noise exposed animals from efficacy pilot study (n=3). Left (treated) and right (untreated). Black arrows indicate the approximate location of a particular frequency along the cochlea as well as the cochleostomy/infusion site. Noise exposure was 4kHz.





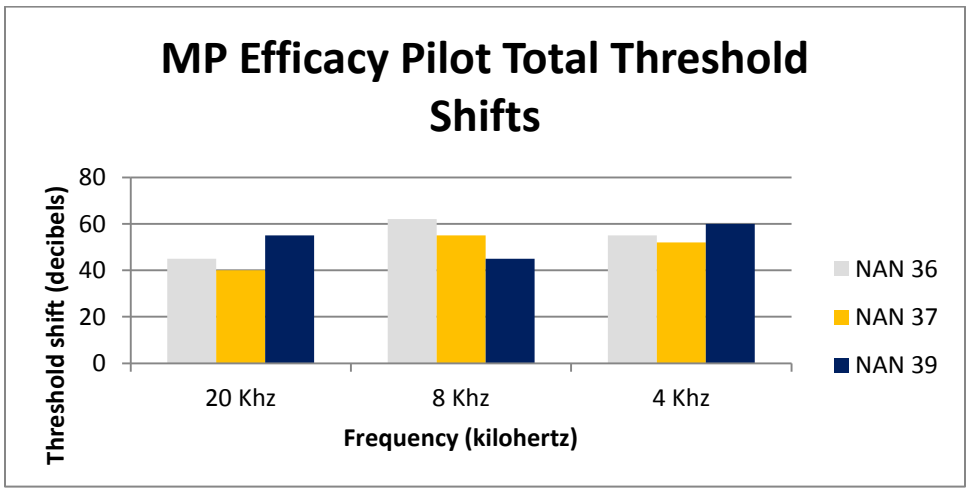


Figure 5-5. Threshold shifts from auditory brainstem responses for efficacy animals. A) Post infusion (5days) B) Post noise (14 days) C) Total.

Table 5-1. Average Counts of Inner Hair Auditory Nerve Connections from Efficacy Experiment

Specimen	ROI 1 (0.4kHz)	ROI 2 (4kHz)	ROI 3 (20kHz)
Treated Ears			
NAN 36	**12.5**	**17**	**12.3**
NAN 37	**11.2**	**14.5**	**13.7**
NAN 39	**14.5**	**13.1**	**10.7**
Treated Average	**12.7**	**14.9**	**12.2**
Untreated Ears			
NAN 36	**12.5**	**15**	**15.5**
NAN 37	**11**	**15.6**	**19**
NAN 39	**11.8**	**13.8**	**19.3**
Untreated Average	**11.7**	**14.9**	**18.0**

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